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The behavior and physiology of protandrous sex change in the cinnamon anemonefish, *Amphiprion melanopus*

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University of Hawaii, 1992

THE BEHAVIOR AND PHYSIOLOGY OF PROTANDROUS SEX CHANGE IN
THE CINNAMON ANEMONEFISH, *AMPHIPRION MELANOPUS*

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ABSTRACT

Protandrous sex change was examined at the histological, endocrinological, and behavioral levels in the Cinnamon anemonefish, *Amphiprion melanopus*. The objectives were a detailed description of sex change in this species in nature and synthesis of changes at these three levels.

The gonads of non-breeding juvenile fish develop initially along a female path. Males have an ovotestis with mature testicular tissue and immature ovarian tissue. Mature females possess no spermatogenic tissue. Sex change was stimulated in the field and gonadal changes were examined histologically. Five days after female removal, no gonadal changes were visible. Extensive spermatogenic maturation and early oogonial proliferation were seen by 10 days. Spermatogenic tissue was nearly completely replaced by previtellogenic oocytes by 20 days and the first indicators of vitellogenesis were observed at 45 days. Sex change was complete by 100 days.

Plasma levels of five steroids were measured in males, females, and at five, 10, and 20 days into sex change. Females had higher levels of androstenedione, testosterone, and estradiol-17β than males and lower levels of 11-ketotestosterone. Cortisol levels were not different in males and females. Androgens decreased in the first ten days of sex change, then increased by 20 days. Estradiol-17β did not increase until 20 days into sex change, after critical early events of ovarian development. Estradiol-to-androgen ratios increased by five days. Cortisol increased over male levels during sex change, peaking at 20 days, then dropped to male levels in females.

Females are the dominant and most aggressive members of social groups and display frequent aggression towards males. Aggression displayed by males

increased significantly within one day following female removal, then decreased gradually. No convincing evidence for a role of androgens or estradiol in increased aggressive behavior during sex change was found either in overall patterns or within individuals. Cortisol appears linked to behavior, but whether as cause or effect is unclear. The pattern of sex-role reversal and androgens in *A. melanopus* is similar to that of polyandrous birds and supports recent theories of the role of steroid hormones in the control of behavior.

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CHAPTER I

INTRODUCTION

Few problems in biology are more fundamental than those relating to sex. The most basic of these is "Why have sex at all?" (Maynard Smith, 1978). Given that an organism will be one sex or the other, the questions which then arise are "Which sex to be?", "How to develop into that sex?", and "How best to reproduce as that sex?". For most vertebrates at least, these latter questions are "asked" once by an individual during ontogeny and gonadal sex becomes a fixed, unmodifiable part of its reproductive strategy. Sexuality is more plastic in many organisms which change functional sex during adult life (Policansky, 1982; Charnov, 1982). This thesis describes several aspects of this change of functional sex in a teleost, the protandrous Cinnamon or Dusky anemonefish *Amphiprion melanopus*.

Sex change is widespread in nature, occurring in plants, invertebrates, teleost fishes, and even one frog (Policansky, 1982; Charnov, 1982; Grafe and Linsenmair, 1989). It is but one example of sexual plasticity from a range of phenomena which include environmental sex determination and simultaneous hermaphroditism. However, in vertebrates, and even among the fishes, sexual plasticity appears to be the exception rather than the rule. It is therefore appropriate to ask: "What is the relevance of sex change to general patterns of sexual determination and development?" Put another way, what can we learn about these phenomena, in fishes and vertebrates generally, from a study of sex change? This is the approach I have tried to take in this work.

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A particularly appealing aspect of protandrous sex change in *A. melanopus* is the proximate control of the process through an individual's social environment. Social control of sexuality in fishes is known primarily from coral-reef species. Socially-controlled sex change was first described in the protogynous basslet, *Anthias squamipinnis* by Fishelson (1970). Reports of other coral-reef species exhibiting this pattern accumulated rapidly (e.g., Robertson, 1972; Fricke and Holzberg, 1974; Warner *et al.*, 1975). Sex change was first described in anemonefishes almost simultaneously in three papers (Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Ross, 1978a). All anemonefish species studied to date mate monogamously and pairs defend as their territory large sea anemones. Females are the largest and behaviorally-dominant members of social groups. Disappearance or removal of the females results in their male mates undergoing sex change to become mature females.

Anemonefishes have been a favorite subject of biologists for some time (Collingwood, 1868; Herre, 1936). They form a specialized subfamily of the Pomacentridae, the Amphiprioninae, comprising 26 species in two genera. Twenty-five of these species are in the genus *Amphiprion* and one is in the genus *Premnas* (Allen, 1972). Interest in their biology was first stimulated by their unusual and typically obligate symbiotic association with large tropical sea anemones (Mariscal, 1970; Allen, 1972; Dunn, 1981). A good deal is now known about their taxonomy and biogeography (Allen, 1972), the relationship with their anemone hosts (Miyagawa, 1989; Fautin, 1991; Godwin and Fautin, 1992), reproductive behavior and biology (Allen, 1972; Ross, 1978a; Yanagisawa and Ochi, 1986; Moyer, 1986), and competition and specialization with respect to host anemones (Dunn, 1981; Fautin, 1986). A number of field studies have also

focused on sex change in the group following its original description (Fricke, 1979, 1983; Hattori, 1991a,b; Ochi, 1989; Ochi and Yanagisawa, 1987). Laboratory studies on the cytological and physiological aspects of sex change in *Amphiprion* have also recently appeared (Brusle-Sicard, 1990, 1991; Latz, 1991; Stahlschmidt, 1988; Stahlschmidt-Allner, 1991).

Social control of sex change provides an important advantage for both field and laboratory experimentation: sex can be precisely manipulated in an adult animal through manipulation of its social environment. This precise control allows the time since the onset of conditions which lead to sex change to be known exactly. Aspects of sex determination and sexual phenotype development can thereby be critically examined in relatively large, adult animals. Additional advantages of the *Amphiprion* , and especially *A. melanopus*, systems include the following: i) As members of the Pomacentridae, they are closely related to both protogynous species (female-to-male change) and gonochoristic species (no adult plasticity in sex). The damselfishes are an extremely well-studied group from the standpoints of behavior and ecology (Thresher, 1984) and the range of sexual patterns they exhibit make them an especially good group for comparative study. ii) The extreme site-attachment of anemonefishes in nature facilitates experimental manipulation in the field and minimizes impacts on the social system from a laboratory setting. iii) *A. melanopus* is relatively common in many areas and is easily captured and cultured. iv) *Amphiprion* species, like other coral-reef fishes, live in a hospitable environment of warm, clear water conducive to field work and behavioral observations.

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This project had two broad aims at its inception. The first was to provide a better integrated description of the sex change process at the histological, endocrinological and behavioral levels than was currently available for any sex-changing species. It was critical that this description be of the process as it occurred in nature and that its temporal aspects be especially well characterized. This would then provide the framework of a model with which hypotheses about behavioral and physiological aspects of sex change could be rigorously tested. I chose *A. melanopus* for the reasons outlined above and because little information was available for protandrous species.

The chapters which follow describe the results from sex change experiments with *A. melanopus* performed in Guam and Papua New Guinea in 1988, 1989, and 1991. Sex change was stimulated in the field by removal of females from mated adult pairs and characterized at the histological, endocrinological, and behavioral levels through observations and recapture of sex-changing individuals after varying periods. Each of the chapters other than the conclusion was written for journal submission; some repetition was therefore unavoidable. Chapter II describes the histological changes in gametogenic tissue and gonadal structure observed over the course of sex change. Chapter III compares the plasma levels of five steroid hormones in unmanipulated males and females with sex-changing fish at points before, during and after critical events of gonadal change. Chapter IV describes behavioral changes and the endocrine correlates of these changes both on the level of overall patterns and within individuals. Each data chapter was written with the broader issues discussed above in mind. These are teleost and vertebrate sex determination in chapters II and III, and behavioral expression and sexual phenotype

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development in chapter IV. The concluding chapter briefly proposes an examination of the phenomenon of sex change and sexuality in teleosts through organization/activation theory (Young *et al.*, 1964; Arnold and Breedlove, 1985), critiques some recent experiments on the physiological control of sex change and suggests important considerations, and finally proposes hypotheses and experimental tests of mechanisms of gonadal and behavioral change during sex change in *A. melanopus*

CHAPTER II

HISTOLOGICAL ASPECTS OF PROTANDROUS SEX CHANGE

Introduction

The phenomenon of sequential hermaphroditism in fishes has attracted
attention as both a fascinating adaptation and for its potential in the study of sex
determination in vertebrates. Teleosts in general, and sex-changing species in
particular, are excellent systems for the study of sex determination for two
reasons (see Shapiro, 1990). A greater range of sexual patterns and degree of
plasticity are seen in fishes than in any other vertebrate group. Adaptations to
terrestrial reproduction in tetrapods have placed constraints on sexual plasticity
not seen in the majority of teleosts (Warner, 1978). Secondly, what may be
viewed as a second sexual maturation takes place in an adult, easily manipulated
animal.

Studies of the ultimate causation of sex change have been successful and
our understanding of the social and ecological factors producing this pattern has
progressed impressively (Warner, 1988; but see Shapiro, 1988). However, the
proximate physiological mechanisms initiating and controlling sex change
remain almost completely unknown.

Gonadal change has been studied at both the light microscope and
ultrastructural levels. These efforts have been primarily for the documentation
of sex change, but physiological mechanisms have also been considered.
Changes occurring in protogynous change have been described for a number of
species (*Coris julis:* Reinboth, 1962; *Thalassoma duperrey:* Nakamura *et al.*, 1989;
Monopterus albus: Chan and Phillips, 1967; *Centropyge potteri:* Lutnesky, in press).

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Protandrous change has been described in two sparids (*Rhabdosargus sarba*:
Yeung and Chan, 1987; *Acanthopagrus schlegelii*: Chang and Yueh, 1990).
Aspects of gonad ultrastructure and development have been described for
laboratory held *Amphiprion frenatus*, a close relative of *Amphiprion melanopus*
(Brusle-Sicard and Reinboth, 1990; Stahlschmidt-Allner and Reinboth, unpubl.
abstr.). A fundamental difference is seen between protandrous and
protogynous forms in the presence and relative maturity of tissue of the
secondary sex in the gonad prior to sex change. In the protandric species which
have been examined, many of the cells destined to be ovarian have entered into
and are arrested in the first meiotic division (primary oocytes) in the initial-phase
(male) gonad (Reinboth, 1988). These oocytes may be topographically separated
from active spermatogenic tissue (delimited gonad type; Sadovy and Shapiro,
1987) or intermingled with it (undelimited gonad type) and are arrested in the
perinucleolar stage of development (see Wallace and Selman, 1981). Reinboth
(1988) notes that the close proximity of gametogenic cells undergoing
spermatogenesis in this gonad type does not appear to interfere with the
differentiation and maintenance of early stage oocytes. Examining gonadal
ultrastructure in *Amphiprion frenatus*, Brusle-Sicard and Reinboth (1990) reported
no separation between the different gametogenic cell types existed in some parts
of the gonad. Protogynous species do not show as advanced a degree of
differentiation of secondary sex tissue prior to sex change. The presence of cysts
of spermatogonia has been reported from some protogynous species (e.g.,
Dascyllus aruanus [Pomacentridae], Sphigel and Fishelson, 1986; *M. albus*, Chan
and Phillips, 1967), but not others (e.g., *Anthias squamipinnis* [Serrranidae]:
Shapiro, 1977; *T. duperrey* [Labridae]: Nakamura *et al.*, 1989). However, the

formation of spermatogonia represents only mitotic proliferation and not the beginnings of meiosis.

The nature of the precursor cells which form the secondary sex gametogenic tissues is not known for any sex-changing species (Reinboth, 1988). Are there germ line cells which are sexually bipotent and can develop in either a female or male direction in the functional initial sex gonad? Alternatively, are there separate germ cell types, one actively producing initial sex gametes and the other relatively inactive until sex change? This will be fundamental to our understanding of the cellular basis of the process as it distinguishes whether sex change represents determination or differentiation.

The anemonefishes form a specialized subfamily within the damselfishes, the Amphiprioninae, and are famous for their unusual symbiotic relationship with large tropical sea anemones. Anemonefishes occupy and defend as their territory various species of anemones in the tropical and warm temperate Indo-Pacific (Dunn, 1981). This unusual association has produced a mating system unique among tropical reef fishes. Nine of the 26 species of anemonefishes (25 *Amphiprion* and one *Premnas* species) have been studied; all are monogamously -paired protandrous hermaphrodites (Fricke, 1983; Fricke and Fricke, 1977; Moyer and Nakazono, 1978; Ross, 1978; Godwin, unpubl. data on *Premnas biaculeatus*). Sex is controlled socially. Females are always the largest and behaviorally-dominant individuals in a group. Disappearance or removal of the female allows sex change to proceed in her mate, a mature male. The dominant subadult individual in a group then matures and assumes the male breeding position.

This study addressed histological aspects of protandrous sex change at the gonadal level in the Cinnamon anemonefish, *Amphiprion melanopus*. This species is found over a wide area of the tropical Western Pacific and associates almost exclusively with the anemone species *Entacmaea quadricolor* (= *Physobrachia douglasi*, see Dunn, 1981). The mating system in this species follows the pattern discussed above and has been described by Ross (1978a,b). *Amphiprion melanopus* is well-suited to the study of sex change in the field because it is relatively common, extremely site-attached, and is easily captured and manipulated. Strong advantages to this system are that experiments can be performed in nature with relative ease and the time since the onset of conditions which lead to sex change can be known precisely.

The work to be reported on here is part of a larger study of sex change in this species also characterizing changes in behavior and plasma levels of gonadal steroid hormones. The objective of this part of the study was to characterize the nature and time course of changes in the gonad both qualitatively and quantitatively. This information is critical to understanding the process as well as providing a "timetable" for interpreting changes in behavior and endocrine physiology.

Materials and Methods

Study locations

The field portion of the study was performed primarily in Guam, Marianas Islands in April-May, 1988 and working from the Christensen Research Institute in Madang, Papua New Guinea (PNG) in June-July, 1989. Some work was also performed at the Lizard Island Research Station, Great Barrier Reef. All manipulations were performed in the field.

Stimulation of Sex Change

Sex change was induced as follows. Both the male and female members of mated pairs were captured with handnets and quinaldine anesthetic. The female was kept while the male was marked, measured to the nearest mm, and returned to his anemone(s). The sex of each individual was known both from their relative sizes (females are the largest and dominant individuals within a group) and from examination of the genital papilla, which is sexually dimorphic in pomacentrids (Thresher, 1984). Additionally, gentle pressure on the abdomen usually caused the extrusion of large orange eggs in females and milt in males. Approximately ten minutes elapsed between capture of both members of a pair and the release of the male back on his anemone. Males were allowed to undergo sex change for periods ranging from 5-100 days before being recaptured and sacrificed to obtain gonad and blood samples (Table 1).

Table 1. Treatment sample sizes and location of manipulations. Days refer to time allowed for sex change after removal of resident female.

Treatment	Guam	PNG	Lizard Is.
4 days	1	-	-
5 days	-	-	2
10 days	5	6	-
20 days	4	4	-
30 days	6	5	-
45 days	-	8	-
100 days	-	-	3

Histological Methods and Quanitification of Gonadal Changes

Upon recapture, gonads were dissected out and preserved in 10% buffered formalin. This was followed by overnight washing in tapwater, dehydration with ethanol, and embedding in paraffin (Paraplast ® brand). Seven micron sections were taken on a standard microtome and slides were stained with hematoxylin and eosin.

The method used to quantify proportions of different gametogenic and other cell types present in the gonad at various sexual stages was based on a stratified random sampling design using "quadrats" (as this term is used in ecology) and is illustrated in figure 1. Gonad length was measured in the embedded paraffin block to the nearest mm. This total length was partitioned into five divisions of equal length and the total number of sections which would be produced by each division calculated based on a 7 μm section thickness. Either one (Guam samples) or five transverse sections (PNG samples) were randomly chosen from each of the five divisions. After sectioning and staining, these sections were placed under the objective of an optical microscope without

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randomly chosen section
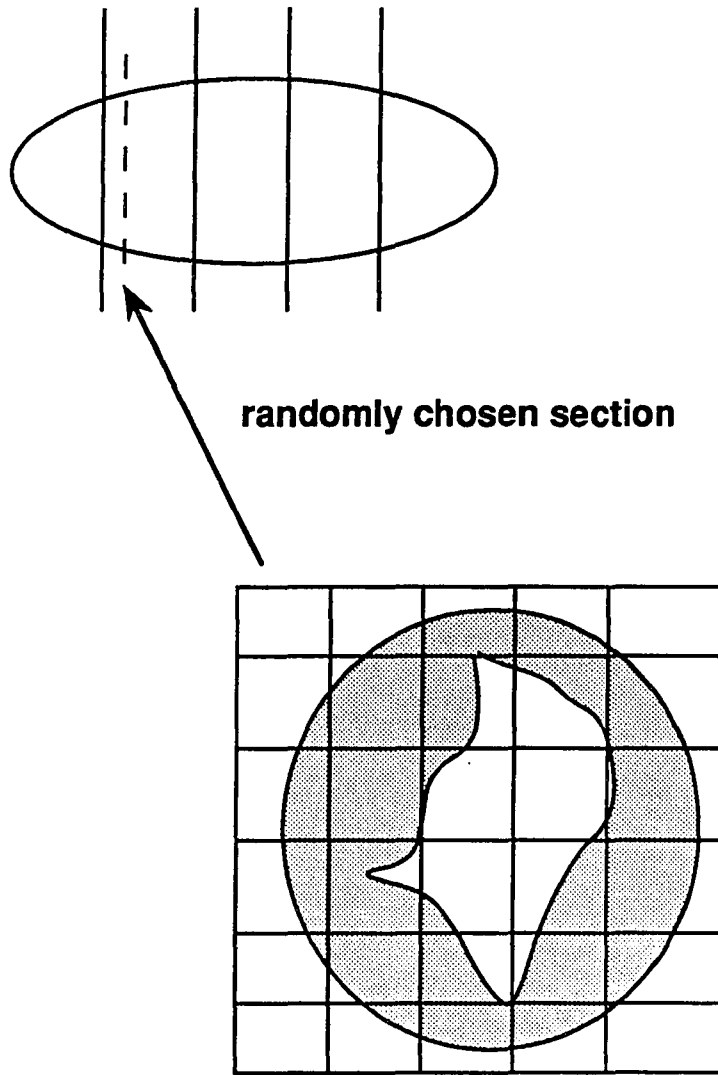
Figure 1. Schematic of quantitative gonadal sampling design.

looking through the ocular, to avoid bias in placement. The ocular was marked with an 11 X 11 grid (121 total line intersections) and the numbers of line intersections falling on oogenic, spermatogenic, and other cell types were counted separately in each of the randomly chosen sections. Points not falling on cells were not counted in the total number of points for a given section. The value for a given gonad was the average of counts from all sampled sections. In this way, total areal coverage of oocytes of various stages as a proportion of both total gametogenic tissue and total gonadal cross-sectional area was calculated. The sampling grid was typically larger than a cross-section through the gonad, the exceptions being where the gonad was a strip of tissue rather than rounded. In any case, the haphazard positioning of the grid should have elimanated any systematic effects of location of the grid on the resulting values.

As a check on the precision of this method, haphazardly chosen sections from five different gonads were quantified five times each with repositioning of the ocular grid each time. The coefficients of variation for oocytes both as a proportion of total gonad tissue (mean CV: 9.5%, range: 6.8-14.0%) and as a proportion of total gametogenic tissue (mean CV: 3.7%, range: 2.2-7.3%) were low and indicated acceptable precision.

In order to allow lumping of data from Guam and PNG samples for statistical analysis, PNG data were treated as follows. As described above, five times as many sections were quantified in gonad samples from the PNG experiments as from the Guam work. To make the data comparable, one of the five sections taken from each of the five divisions of the gonads in the PNG samples was randomly selected and this smaller number of total sections from the gonads was then used in calculating areal coverage of oocytes. This measure

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of progress through sex change was used for individuals from the 10, 20, 30, and 45 days after female removal treatment. One four day and two five day animals (from Guam and Lizard Island respectively) were not quantified.

Unmanipulated males, females and juvenile, non-breeding individuals were captured in Guam and Papua New Guinea and processed for histological as described above. The six juveniles ranged in size from 31-44 mm SL.

Classification of gametogenic stages follows Wallace and Selman (1981) for ovarian tissue and Nagahama (1983) for spermatogenic tissue.

Statistical Analysis

Quantitative measures of gonadal histology were compared either parametrically with analysis of variance or non-parametrically (where extreme heterogeneity of variance violated the assumptions of ANOVA) using the GLM and NPAR1WAY procedures respectively in SAS (SAS Institute, Cary, N.C.). Treatments differences, where indicated, were explored with Tukey's HSD multiple comparison procedure in GLM and Tukey-type *a posteriori* multiple comparisons performed by hand calculation following NPAR1WAY (Zar, 1974).

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Results

Manipulated male *A. melanopus* ranged in size from 56 to 83 mm standard

length (PNG: mean = 63.5 ± 5.4mm SL [SD], range = 56-77, n=29; Guam: mean =

73.5 ± 7.8mm SL , range = 59-83, n = 11). Their female mates prior to

manipulations were always at least the same size or typically larger (PNG:

mean = 69.5 ± 6.7mm SL, range = 59-83, n = 29; Guam: mean = 78.4 ± 8.0mm SL,

range = 69-96, n = 11). The minimum sizes for function as either a male or female

were not found. All of the females in the above groups had large vitellogenic

eggs at the time of their removal. The size difference between members of mated

pairs was relatively small (PNG: mean = 5.44 ± 3.78mm SL, range = 0-15; Guam:

mean = 4.82 ± 4.73, range = 0-15).

Male, Female and Juvenile Gonadal Histology

The gonad in juvenile, non-breeding individuals consisted primarily of

immature ovarian tissue (fig. 2a). Primary oocytes formed the bulk of the gonad

and spermatogenic tissue was not present. It appeared that spermatogenic tissue

does not develop extensively unless an individual assumes the male breeding

position.

Mature, functioning male *A. melanopus* possess an ovotestis in which

mature testicular tissue and immature ovarian tissue are both present and

incompletely delimited (fig. 2b). The testicular portion of the gonad is of the

unrestricted spermatogonial type (Grier, 1981). Spermatogenic tissue in various

stages is found around the periphery of the gonad and is organized as cysts of

cells of the same stage of maturity. The cysts of spermatogenic tissue are

separated by only a thin cellular barrier. Primary growth phase oocytes in the

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early and perinucleolar stages of development form a layer two to three cells thick around the prominent central lumen in the rostral part of the gonad and along the lateral edge posteriorly. Posteriorly, the lumen opens and the gonadal lobes are bands of tissue with spermatogenic tissue medial and oogenic tissue lateral. No differences in the degree of maturity of spermatogenic or oogenic tissue were seen along the length of gonads. Spermatozoa collect in sperm ducts which run along the periphery of the gonad rostrally and along the medial side more posteriorly. Sperm collect posteriorly in a sinus which is continuous through a narrow connection with the duct of the genital papilla.

Considerable size variation was seen between primary oocytes in the functional male, perhaps suggesting a continuing production and degeneration even in male functioning individuals (evidence for this is given in Brusle-Sicard and Reinboth [1990] for the closely related *A. frenatus*). Oocytes maturing past the previtellogenic stage were not observed in individuals with active spermatogenesis. Also found in the gonads of functional males and early stage intersexual animals are bodies without discernible structure approximately the size of large primary oocytes. These structures stain yellow-brown in hematoxylin and eosin and are similar to "yellow-brown bodies" described in the secondary sex phase of several protogynous teleosts (see Sadovy and Shapiro, 1987). These "yellow-brown" bodies were always found in the midst of spermatogenic tissue in the gonad and separated from primary oocytes.

Functional females show no evidence of either spermatogenic tissue or the prominent central gonad lumen seen in males (Fig. 2c). Oocytes in all stages of maturity were found in the females examined. Luminal spaces are seen between lobes of the ovarian tissue. These lobes attach to the ovarian wall, but often

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Figure 2a-c. Gonadal structure of a) juvenile, b) male, and c) female *Amphiprion melanopus* (cross-sections shown, scale bar = 100 μm, PO: previtellogenic oocyte, SP: spermatogenic tissue, GL: gonadal lumen, EV: early stage vitellogenic oocyte, YV: yolk vesicle stage oocyte).

A

PO

B

PO

GL

SP

C

YV

EV
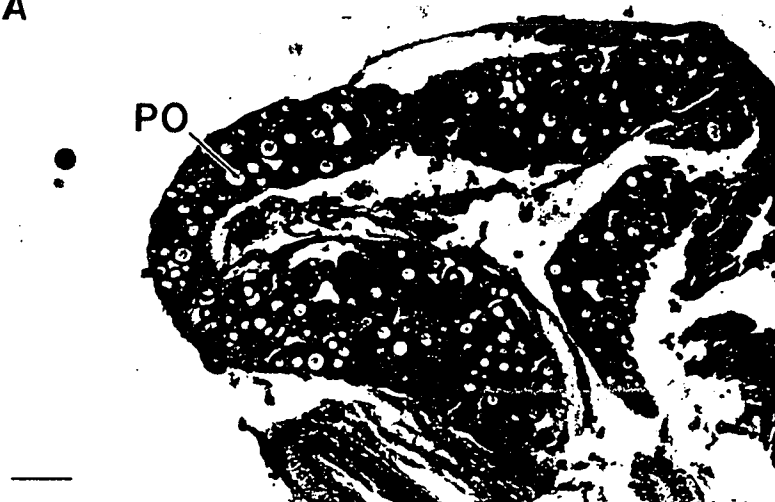
appear isolated in cross-section. Posteriorly, the duct of the genital papilla expands to encompass the lower lobes of the ovary and a continuity with the ovarian luminal spaces can be seen. Oocyte development appears to be group synchronous (Wallace and Selman, 1981), and this species is known to spawn on an semi-lunar cycle. However, the types of counts necessary to distinguish between this pattern and asynchronous oocyte development were not performed.

Changes in Gametogenic Tissue with Sex Change

In the two individuals allowed to change sex for five days at Lizard Island and single individual allowed to change for four in Guam, no histological changes from the male gonadal structure discussed above were evident. However, dramatic changes in the organization of the gametogenic tissue occurred in all individuals examined by the ten day point of sex change (fig. 3a and b). Nearly all spermatogenic tissue in the gonad appeared to have matured into the spermatzoan stage, while no early spermatogenic stages were observed developing. The cyst structure of the testicular part of the gonad was much less evident and the partitions between tissue compartments containing spermatogenic tissue were thickened as compared to those seen in unmanipulated males (compare with fig. 2b). These partitions now generally consisted of a central flattened cell layer with layers of undifferentiated cells on either side. The central flattened cell layer was similar in appearance and thickness to that seen in unmanipulated males. The cells on either side, which accounted for the increased thickness of the tissue partitions, were similar in appearance to oogonia seen in females. These were rounded cells with a large nucleus to cytoplasm ratio and are interpreted to be proliferating oogonia.

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Variation in the degree of proliferation of these putative oogonia was found between individuals. Individuals in which i) more of the gonad and total gametogenic tissue was accounted for by primary oocytes, and ii) the maturation of the spermatogenic tissue was more advanced (few spermatids evident) also showed a greater proliferation of these putative oogonia. Interestingly, these cells appear to develop in the same location as cysts of spermatogenic tissue formed prior to the initiation of sex change. There was no change in either the number or appearance of primary oocytes surrounding the central lumen as compared to males in ten day intersexuals.

Twenty days into sex change, nearly the entire peripheral area of the gonad in all individuals was occupied by primary oocytes (fig. 3c). This suggests that these oocytes developed from the putative oogonia seen on either side of the tissue partitions in ten-day intersexes. The obvious tissue partitions present at ten days after female removal had disappeared by this point. Oocyte maturation beyond that seen in unmanipulated males was still not evident. Most oocytes were in the perinucleolar stage. Isolated areas of spermatogenic tissue, typically in the spermatozoan stage were found but were rare in most individuals. Gonadal histology at 30 days after female removal was similar to and not distinguishable from 20 day animals in terms of oocyte development and general appearance of the gonad. Spermatogenic tissue was still rarer at 30 days after female removal.

Figure 3a-c. Gonadal structure of a) 10 day intersexual, b) close-up of 10 day intersexual, and c) 20 day intersexual *Amphiprion melanopus* (cross-sections shown, scale bar = 100 µm, TP: tissue partition, SZ: spermatozoa, PO: previtellogenic oocyte, OO: putative oogonia).
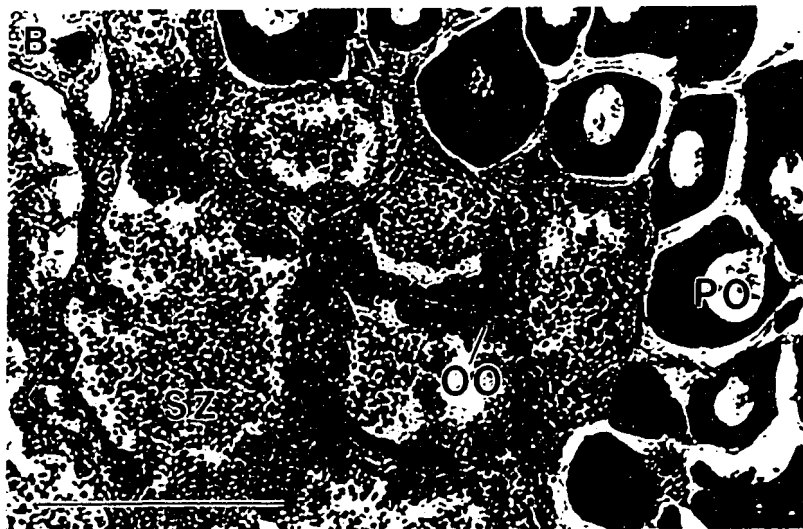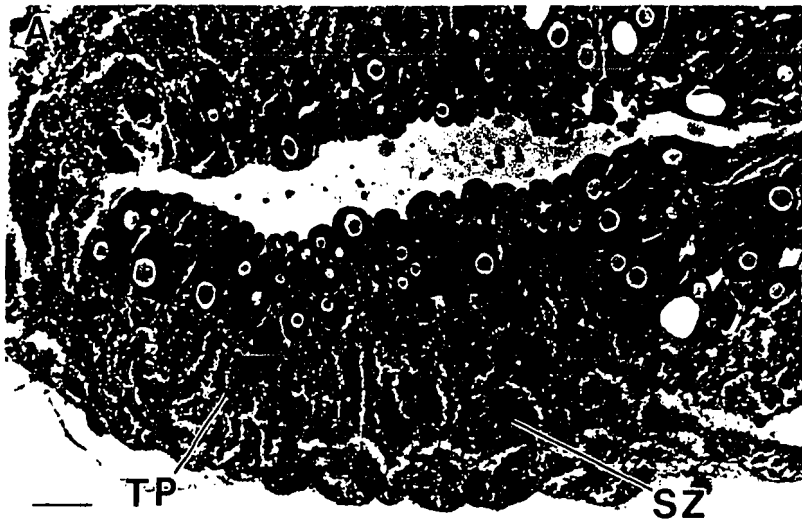
Evidence of vitellogenesis was first seen in the 45 day treatment, but in only one of eight fish. This fish had oocytes developed to the yolk vesicle stage. The beginning of vitellogenesis is a significant event as yolk deposition is an indicator of mature female function and is taken here as an indicator of completion of sex change. The three fish allowed to change sex for 100 days at Lizard Island showed complete sex change. All stages of oocyte development were present, gonadal structure was indistinguishable from that of functioning females examined, and their genital papillae had assumed the female form. It is not known whether these fish had begun spawning as females.

Changes in Lumen Structure

Changes to the structure of the gonadal lumen occurred late in sex change relative to changes in gametogenic tissue. The transformation of the lumen from the male form to that of females occurred by progression of the invagination of lateral, ovarian sides of the gonadal lobes and closure to form a central lumen described above for males. This process was complete and the luminal space continuous with the gonoduct in only one of the 30 day and two of the 45 day animals. As lumen closure progressed, sperm ducts in the periphery of the lobes were occluded by development of primary oocytes, becoming progressively less obvious and disappearing completely in females.

Quantitative Changes in Gonadal Histology

As with cytological changes in the gonad, change in the proportion of the gonad accounted for by oocytes changed most dramatically in the first twenty days of sex change (figure 5a and b). A slight increase in the proportion of total gametogenic tissue accounted for by oocytes over that seen in males was suggested at ten days after female removal. By twenty days into sex change, a

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mean of 90% of total gametogenic tissue was oocytes. Spermatogenic tissue was nearly absent by thirty days in ten of eleven individuals examined, being found only in small cysts in the spermatozoan stage when present. This was also the case at 45 days, but cysts of spermatozoa were still rarer. One individual allowed to change sex for thirty days did still have substantial amounts of spermatogenic tissue at the end of this period (20.2% of gametogenic tissue). The only significant differences between treatments in oocytes as a proportion of total gametogenic tissue were these: the male and ten day treatments differed from the 30 day treatment (Kruskal-Wallis X_2 approximation = 21.48, df = 3, p<0.0001, *a posteriori* comparisons: p<0.01). The 45 day treatment group was not included in this analysis since the variance in oocytes as a proportion of gametogenic tissue was very small and the assumption of equal variances would have been severely violated.

The proportion of total gonadal tissue accounted for by oocytes increased significantly over male and ten day intersexual values by 30 days into sex change and remained higher at 45 days (ANOVA F=3.10, df=4,35, p<0.05, *a posterori* comparisons: p<0.05).

Considerable variation was seen between individuals in the rapidity of replacement of spermatogenic tissue with oocytes. This was especially evident in the ten day treatment, but was also seen in the twenty day treatment. This variation was not obviously correlated with body size or number of smaller fish resident in the sex-changing individual's group.

Caudal   Rostral

▨ Ovarian Tissue

▨ Testicular Tissue
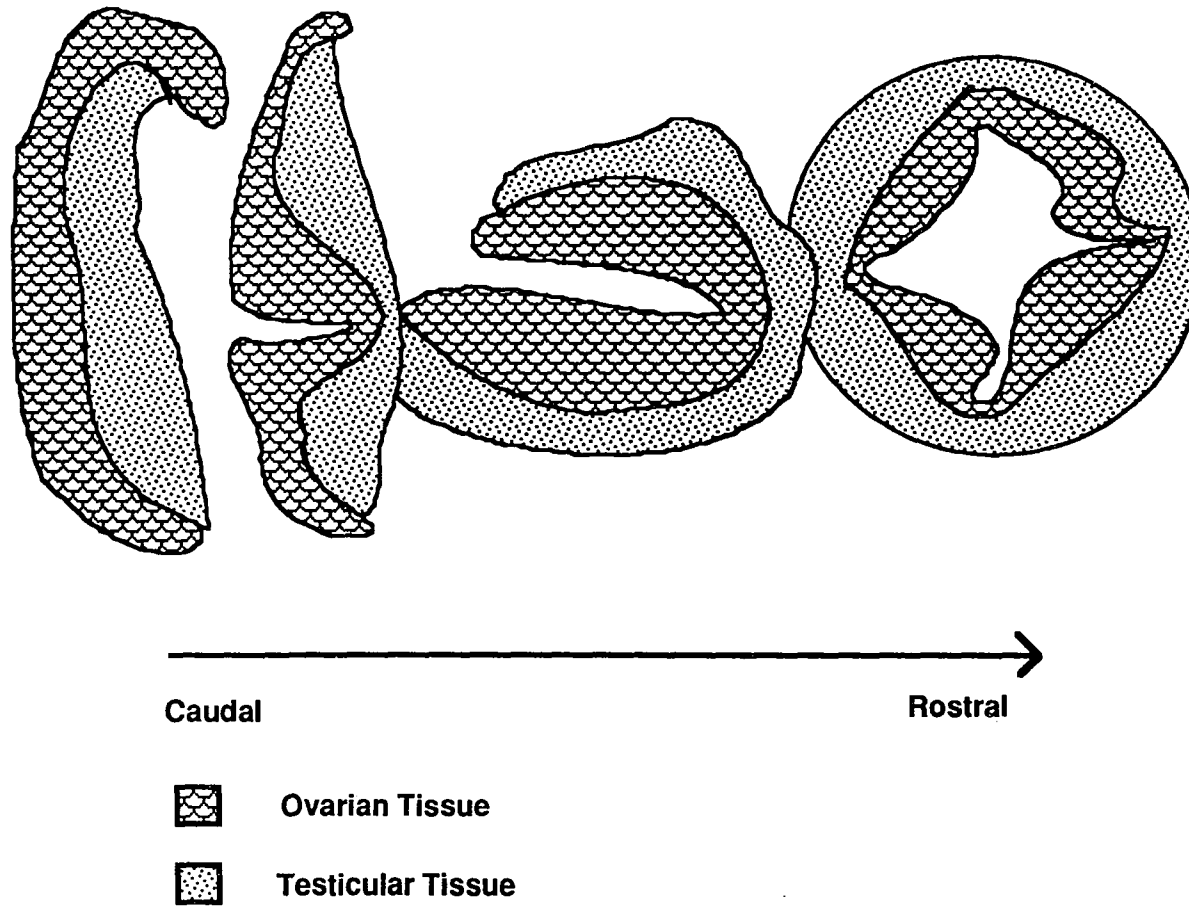
Figure 4. Gonadal lumen structure in males shown in caudal to rostral transverse
sections.
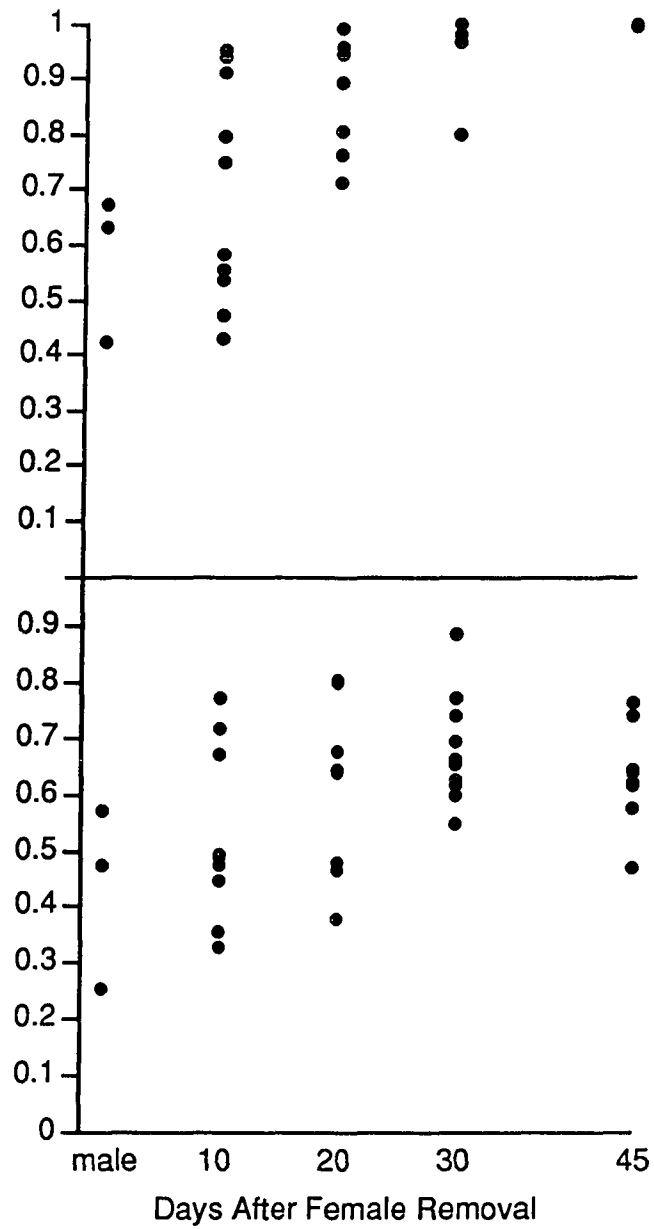
Figure 5. Oocytes as a proportion of (a) total gametogenic tissue, and
(b) total gonadal tissue in *Amphiprion melanopus* at various
points in sex change.

Discussion

Duration of the sex change process

The duration of the sex change process in *A. melanopus* found here, with the complete transition from male to female function taking place in between 45 and 100 days, is in general agreement with findings for other species of *Amphiprion*. The shortest reported completion of sex change for an *Amphiprion* is species is 26 days for an individual *A. bicinctus*, while seven other individuals completed sex change in less than 145 days (Fricke, 1983). Two other species, *A. akallopisos* and *A. clarkii*, have been observed to change sex in less than 63 days and less than 5-6 months respectively (Fricke and Fricke, 1977; Moyer and Nakazono, 1978). A male *Premnas biaculeatus* placed in an aquarium for 100 days with two smaller conspecifics at the Lizard Island Research Station by the author assumed a darkened body color, as is characteristic of females in this species (Wood, 1980), and its genital papilla morphology was female.

Sex change in *A. melanopus* appears to be slower than that reported for most species with socially controlled protogynous sex change, including the related pomacentrid *Dascyllus aruanus*. In groups of *D. aruanus* from which the dominant male had been removed, sex change was either complete (15) or intersexes were present (3) in 18 of 20 after 50 days (Coates, 1982). Protogynous change also appears to be much faster than protandrous change in sparids (Reinboth cf. Atz, 1964). Sadovy and Shapiro (1987) summarize reported durations for completion of sex change for 17 protogynous species. The process is definitely more rapid in nine of these species and slower in one species; the data are insufficient for comparisons with seven others.

Differences in the rate at which species change sex, particularly between protogynous and protandrous forms, may reflect both differences in the complexity and energetic costs of morphogenetic changes associated with gonadal transformation as well as differences in selective pressures on rate of sex change imposed by mating system. In addition to the energetic costs of gonadal change, the "down time" during transformation represents lost reproduction (usually) and apparently also a period of relative vulnerability to territory takeover by a secondary-sex individual from outside a sex changer's social group. Ross (1990) proposes that the threat of harem takeover, with the consequent loss of expected reproductive success, likely presents a strong selective pressure for rapid assumption of male function in many protogynous species. In the protandrous *A. frenatus*, Brusle-Sicard and Reinboth (1990) suggest that an apparently continual production of primary oocytes in males may enable them to change sex more rapidly. During these experiments, territory takeovers by females from outside manipulated groups were observed after removal of dominant females. In contrast to the situation in protogynous species however, Godwin (in prep.) argues that territory takeover by an individual larger than the resident male in protandrous *Amphiprion* represents a benefit and selection for rapid sex change in not likely to be as important as in protogynous species. Fecundity is closely related to body size in fishes and an individual male anemonefish should realize an immediate increase in reproductive output by pairing with a larger individual instead of changing sex.

Pattern of gonad development

    Amphiprion melanopus is normally functionally protandrous but developmentally protogynous, at least until the initiation of mature female function. Oocytes appear early and are a prominent part of the gonad in both juveniles and functional males. Recent work by two groups indicates that *Amphiprion* are dygynic: two developmental pathways to mature female function are exhibited (Stahlschmidt-Aller and Reinboth, unpubl. abstr.; Shapiro and Fautin, unpubl. abstr.). The largest juveniles in a group may mature and assume male function in the presence of a mature female or develop directly as a female with no intervening male phase if no larger individuals are present. Hattori and Yanagisawa (1991) have described similar flexibility for *A. clarkii* in a temperate habitat in Japan. This may be adaptive in two situations. i) Social groups containing only small individuals are common in nature (pers. obs. for *A. melanopus, A. clarkii,* and *A. chrysopterus*). ii) *Amphiprion* males have been observed to abandon their territories following disappearance or removal of females both in the course of these studies (Godwin, in prep.) and in Japanese populations of *A. clarkii* (Ochi and Yanagisawa, 1987; Ochi, 1989), leaving the largest juveniles as the dominant individuals in a group. Development and maturation of testicular tissue with no female present to mate with would be a waste of resources in either of these situations.

    The presence of primary oocytes in developing or functioning testes is not unusual in fishes. A number of gonochoristic fishes in various groups appear to regularly develop primary oocytes during sexual differentiation in gonads destined to be male. This pattern has now been described for cyprinids (Takahashi, 1977; Takahashi and Shimuzu, 1983), cichlids (Peters, 1975;

Naish and Ribbink, 1990), chaetodontids (Tricas and Horimoto, 1989), and sparids (Buxton and Garratt, 1990) and has been variously termed secondary gonochorism, rudimentary hermaphroditism, and pre-maturational sex change (a standardization of terminology is clearly in order here). In the cichlid *Pseudotropheus lombardoi*, primary oocytes are retained from this juvenile stage into the adult testis (Naish and Ribbink, 1990). Francis (1992) suggests that female development is the "default" pattern for teleosts, with gonads developing along a female pathway unless male development is somehow induced. The developmental pattern seen in *A. melanopus* supports this interpretation, being similar up to the change to female function and loss of all vestiges of spermatogenic tissue. It may also indicate that protandry is secondarily derived from protogyny in this group, perhaps in association with the symbiosis with anemones.

Cytological changes during sex change

The primary sex (male) tissue present in the gonad at the onset of sex change appears to undergo a rapid maturation following female removal rather than the immediate degeneration which is characteristic of protogynous species. Both the extent of the area of the gonad occupied by the mature spermatid stage and rarity of less-mature spermatogenic stages point to a maturation of the spermatogenic tissue present at female removal rather than a disappearance of all but the most mature stages. The general lack of cyst-like structure is also consistent with maturation and breakdown of cyst walls seen in other teleosts (Grier, 1981). This indicates that the development from primary spermatocyte to at least the spermatid stage can occur in ten days or less.

The pattern of change in primary sex tissue seen here differs strikingly from that observed in protogynous species. One criterion established for strong inference of functional protogyny in fishes is the presence of degenerating oocytes (atresia) and proliferating spermatogenic tissue in the same gonad (Sadovy and Shapiro, 1987). In the protogynous sequential hermaphrodites which have been examined, oocyte atresia is the first histological indicator of sex change and is followed only later by development of spermatogenic tissue (e.g., *Thalassoma duperrey*: Nakamura, *et al.*, 1989). In contrast, spermatogenic tissues in *A. melanopus* appear to proceed to terminal differentiation before being replaced almost completely at twenty days into sex change by primary oocytes. The continued development of spermatogenic tissue proceeds only microns from what are postulated here to be proliferating oogonia at the ten day point in sex change (Fig. 3a,b). These cells may even be in physical contact as has been observed in males of *A. frenatus* (Brusle-Sicard and Reinboth, 1990). This differentiation may have taken place prior to proliferation of the putative oogonia, however. Extensive proliferation of oogonia was seen in gonads where all or nearly all spermatogenic tissue had matured at ten days, suggesting these are sequential rather than concurrent events. It appears that spermatogenesis proceeds once begun, but that after the initiation of sex change development is instead directed along a female path. Whether this reflects differing influences on a single bipotential germ cell before and after the initiation of sex change or the activation of a oogenic precursor and inactivation of a spermatogenic precursor is not known. This problem is discussed further below.

The primary delay in the assumption of mature female function in *A. melanopus* was the lag between development of oocytes throughout the gonad

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and the further maturation of these oocytes. In functional males, most of the oocytes lining the lumen of the ovotestis are in the perinucleolar stage of development. This is the stage just prior to vitellogenesis. However, no signs of further maturation were evident in either the oocytes which were presumably lining the lumen at female removal or those which developed in the periphery of the gonad until 45 days after female removal, and then in only one of eight individuals. This pattern suggests that formation of the early oogenic stages is not the rate determining step in sex change. Rather, the onset of vitellogenesis appears to limited by some other process.

Vitellogenesis requires the participation of other systems including secretion of pituitary gonadotropins and synthesis of vitellogenin in the liver (De Vlaming et al., 1980; Ng and Idler, 1983). This may explain why continued differentiation of spermatogenic tissue is observed after the initiation of sex change in A. melanopus, while oocyte atresia begins immediately in protogynous species. The primary growth phase of oocytes up to a "critical size" is not dependent on pituitary hormones in teleosts (Wallace and Selman, 1981). The apparent turnover of primary oocytes in males, as suggested by Brusle and Reinboth (1991) for A. frenatus based on ultrastructural observations, and proliferation of this stage in transitional individuals is probably not dependent on pituitary influences either. Synthesis of yolk materials in the liver is dependent on estrogenic stimulation. This stimulation requires both circulating estrogens and estrogen receptors in liver tissue. Circulating levels of estradiol-17β are known to be higher in females than males of some protogynous species (Nakamura et al., 1989; Cardwell and Liley, 1991a). Plasma levels of this hormone are significantly lower in both males and early stage intersexual

individuals than in mature females in *A. melanopus* (Godwin and Thomas, in prep.).

Physiological Models of Sex Change

In teleosts, as in other vertebrates, primordial germ cells migrate to the developing gonads and form the gametogenic tissues of the adult. That these cells are sexually bipotential at this stage is suggested by the ability to completely manipulate phenotypic sex by administration of gonadal steroid hormones before and during sexual differentiation in a number of gonochoristic species in various families (see reviews in Yamamoto, 1969; Hunter and Donaldson, 1983; Yamazaki, 1983). Yamamoto (1969) postulated that steroid hormones are the primary sex inducers in fishes based largely on this evidence. This developmental plasticity is lost after sexual differentiation and there is no evidence that hormone treatment clearly and unambiguously changes gonad development after this point. Whether sex change in adult fishes represents retention of this juvenile plasticity, and therefore a neotenous character, or the differential activation of separate oogenic and spermatogenic stem cell lines in the adult gonad remains an open question in many species (see Francis, 1992 for further discussion of this point).

In *A. melanopus*, oogonia and spermatogonia arise in the same location in the gonad as nearly as can be determined from these experiments. This is weak evidence for a bipotential germ cell model and contrasts with what is seen in fishes with delimited gonads. On the contrary, the apparent simultaneous development of both spermatogenic and oogenic tissues in the functional male may argue for two separate gonial cell lines. Lack of pituitary or sex steroid stimulation for the oogenic portion of the gonad while the testicular portion of

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the gonad is stimulated could produce "male" function until the point of sex change. This issue will be taken up in a later paper describing sex steroid profiles over the course of sex change in this species (Chapter III; Godwin and Thomas, in prep.). In protandrous sparids, oogenic and spermatogenic precursors migrate to different areas in the gonad early in development and initiate gametogenesis at at different points in the life history of an individual. The earliest stages of gonad development were not examined in *A. melanopus* and a similar pattern cannot be ruled out despite the lack of distinguishable or delimited separate precusor tissues in adults.

CHAPTER III

STEROID PROFILES AND PROTANDROUS SEX CHANGE

Introduction

Gonadal steroids have been a primary focus in investigations of the physiological basis of sex determination in vertebrates. Jost (1970) proposed a hormonal theory of sex determination for mammals which invoked an organizing role for gonadal steroids acting on sexually bipotential structures in early development. Bogart (1987) extended this theory to propose a parsimonious general explanation for vertebrate sex determination through control of the aromatization of androgens to estrogens. Non-traditional model species such as reptiles exhibiting temperature-dependent sex determination and sex change in fishes have been recognized not just for their potential to improve understanding of sex determination within their respective groups, but also sexual development generally (Bull, 1983; White, 1991; Reinboth 1988; Shapiro 1990). Sex change in teleosts is particularly useful since critical events of sexual phenotype development take place in easily-manipulable adult animals.

Based primarily on the observation that phenotypic sex in teleosts can often be completely manipulated by exogenous sex steroids early in development, Yamamoto (1969) postulated that these hormones were the primary natural sex inducers in fishes. Besides the successful manipulation of phenotypic sex, Yamamoto's hypothesis has received support from studies which demonstrated sex differences in levels of gonadal steroids during development (Feist et al., 1990). Evidence for a role of gonadal steroids in teleost sex change has been more equivocal (see reviews in Cardwell and Liley, 1991a; Francis, 1992). Precocious sex change has been stimulated by administration of

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exogenous gonadal steroids in a number of species (Stoll, 1955; Reinboth, 1962; Cardwell and Liley, 1991a) but not others (Tang *et al.*, 1974a). Circulating gonadal steroid patterns have provided correlative evidence for a role of steroid hormones in sex change in some cases (Nakamura *et al.*, 1989; Cardwell and Liley, 1991a; Cochran and Grier, 1991), while no clear pattern emerged in other species (Yeung and Chan, 1987a,b).

The importance of studying phenomena such as reproductive behavior and sex change in the natural setting has been discussed by others (Cardwell and Liley, 1991a; Pankhurst, 1990). Conditions of captivity may exercise strong and unpredictable effects on both behavior and endocrine physiology. Ideally, reproductive behavior and its endocrine correlates should be examined under natural conditions prior to performing manipulative studies in the laboratory (Pankhurst, 1990), although such an approach may not be feasible for some species. Cardwell and Liley (1990) note that the warm, clear tropical waters inhabited by coral-reef fishes provide a near ideal environment for this type of research. For studies of sex change, the relative lack of reproductive seasonality in tropical habitats eliminates a confounding variable which can complicate interpretation of endocrine results in temperate species.

Examined in this paper are steroid profiles in a wild population of the protandrous anemonefish *Amphiprion melanopus*. *Amphiprion melanopus*, like other anemonefishes, is obligately symbiotic with large tropical sea anemones and forms long-term monogamous pair bonds. *Amphiprion melanopus* is found over a large area of the tropical western Pacific and breeds year-round on an approximately semi-lunar cycle (Ross, 1978a). Groups of *A. melanopus* occupy and defend clusters of the anemone *Entacmaea quadricolor* as their territory.

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These groups display linear, size-dependent dominance heirarchies in which females are the largest and dominant individuals. Sex is controlled socially. Male-functioning fish are the second-largest individuals in social groups and change sex upon disappearance of the female. Other, smaller resident fish have immature gonads and do not participate in breeding.

Godwin (Chapter II, in prep.) has described the gonadal histology of juveniles, males, and females as well as the sequence of changes which occur during sex change in this species. These changes are summarized briefly here as they pertain to the strategy for blood sampling in this study. Male gonads contained both mature spermatogenic tissue and immature ovarian tissue (pre-vitellogenic oocytes). Five days into sex change (post-female removal), histological changes in gonad structure were not visible at the light microscope level. The first visible changes were seen at ten days into sex change: the spermatogenic tissue present had matured and proliferating oogonia were visible in most individuals. Spermatogenic tissue is nearly absent and pre-vitellogenic oocytes occupied the bulk of the gonad by 20 days after the initiation of sex change. The gonad may be considered an immature ovary at this point. The first signs of vitellogenesis were not seen until 45 days into sex change. The study to be described here concentrated on the early period of dramatic change in gonadal structure. Blood samples for this study were taken at five, ten, and 20 days.

The important advantages of this system are that natural sex change can be experimentally induced with relative ease with the time since the onset of sex change known precisely, and blood sampling can then be performed prior to, during, and after critical histological changes in gonadal structure take place.

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The hormones selected for measurement were the gonadal steroids androstenedione (Ad), testosterone (T), 11-ketotestosterone (11-KT) and estradiol-17β (E$_2$), and the corticosteroid cortisol (F). Yeung and Chan (1987a,b) found Ad was the only gonadal steroid to show an apparent relationship to sex change based on *in vitro* steroidogenesis and plasma levels in the protogynous *Monopterus albus* and the protandrous *Rhabdosargus sarba*. T, 11-KT, and E$_2$ have been found to be important for reproductive function in a number of fishes (Fostier *et al.*, 1983). Cortisol is the primary stress hormone in teleosts (Mazeaud, 1977), but its function in reproduction is not well understood. Differences in circulating levels of cortisol over semi-lunar and seasonal spawning cycles (Bradford and Taylor, 1987; Wingfield and Grimm, 1977; Lamba *et al.*, 1983), influences on parturition in guppies (Venkatesh, et al., 1991), correlations with social interactions and status (Hannes, 1985, 1986; Scott, 1980), and *in vitro* effects on gonadal steroidogenesis (Safford and Thomas, in prep.; Carragher and Sumpter, 1990) have all been described. Because of the intimate relationship between social interactions and the control of sexuality in sex-changing fishes, information on cortisol may contribute to an understanding of the physiological mechanisms of sex change.

This paper describes the pattern of circulating steroids during protandrous sex change in *A. melanopus* and relates this pattern to concurrent histological changes in the gonad. The behavioral endocrinology of sex change in *A. melanopus* will be discussed elsewhere (Chapter IV, in prep.).

Materials and Methods

Study location and habitat characteristics

All field work reported on here was performed from the Christensen Research Institute (CRI) near Madang, Papua New Guinea (lat., long) during July-August, 1991. Groups of *A. melanopus* were located in the field on small coral patch reefs and along the outer barrier reef in 1-8 m water depth within 2 km of CRI.

Handling, capture methods, and stimulation of sex change

Fish were captured underwater during daylight hours (0700-1800h) either snorkeling or with SCUBA using handnets and quinaldine anesthetic (2-methyl quinoline). Sex change was induced in male *A. melanopus* through removal of their female mates. Both members of a pair were captured. Males were individually marked (fin-clipped), measured to the nearest mm (standard length), and released back on their anemones within 15 minutes. These males were later recaptured after being allowed to undergo sex change for periods of five, 10, or 20 days. Females were maintained in aquaria with running seawater until the termination of experiments and released back onto anemones in the field. Sex can be reliably determined in the Pomacentridae by examination of the sexually dimorphic genital papilla (Thresher, 1984). Additionally, gentle pressure on the abdomen usually causes extrusion of either milt or eggs, verifying sexual identifications.

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Sampling and treatment of blood

All blood sampling was performed during daylight hours (0700-1800 h). Blood was collected immediately after capture and transport of fish to a small boat. The time elapsed from when the diver approached to within 2m of a colony to initiate capture (initial approach) until blood began to flow into the syringe was recorded for each fish. Fifty to 250μl of blood was drawn from the caudal vein using a heparinized 25 gauge needle and 1 ml disposable plastic syringe. Samples were stored on ice until return to the laboratory (2-4.5 h), then centrifuged at 780 g for ten minutes. Plasma was drawn off and stored at -20°C or colder until assayed. Females held in tanks during sex change experiments were sampled in a similar manner for generation of a plasma pool after they had been held for between one and three weeks. All of these fish appeared healthy and fed normally.

Column chromatography.

Steroids of interest were separated by partition chromatography on celite microcolumns prior to measurement by radioimmunoassay. Solvents (hexane [Fisher H 302-1], ethyl acetate [J.T. Baker 9281-01], isooctane [Mallinckrodt 5603], ethylene glycol [Aldrich 10,246-6], propylene glycol [Aldrich 24,122-9]) were purchased from suppliers and not redistilled before use.

Columns were prepared as follows. Celite:water and celite:glycol mixtures were mixed using the method of White and Thomas (in press). Water or glycol/water mixtures (see below) were added to celite and, stirred briefly, and put in a clear plastic bag. The bag was filled with N_2 gas and shaken vigorously for one minute. The mixture was then compressed on a table top using a rolling pin (a 10 ml pipet). This procedure was repeated twice.

"Long" columns were prepared using 1.5 ml of packed celite: ethylene glycol:propylene glycol:water mixture (6:1:1:1; w:v:v:v) on top of a 0.5 ml packed celite:water "water trap" (3:1; w:v) in 5 ml disposable serological pipets (this column design is used in John Wingfield's laboratory at the University of Washington).

Samples were thawed and aliquoted for assay in volumes of 50 µl or less. Approximately 500 cpm (700 cpm for E_2) of radiolabelled tracer of each steroid to be measured was added to samples for estimation of extraction efficiency and allowed to equilibrate for at least one hour. Samples were extracted with 2.0 ml hexane/ethyl acetate (v:v), evaporated to dryness in a Savant Instruments SVC200 Speedvac connected to a RT4104 refrigerated condensation trap or under a gentle stream of nitrogen gas at 40°C, resuspended and applied to columns in 0.5 ml 10% ethyl acetate in isooctane (v:v), and rinsed with a further 0.5 ml of this mixture. Five steroids were then eluted from columns in the following sequential 4.0 ml fractions (figures in parentheses refer to proportion of ethyl acetate in isooctane, v:v): androstenedione (2%), testosterone (10%), estradiol-17B (20%), 11-ketotestosterone (30%), and cortisol (55%). Overlap was less than 5% in all fractions. 11β-hydroxytestosterone elutes primarily in 40% ethyl acetate in isooctane in these columns (Wingfield, pers. comm.) and was not collected here. Some overlap into the 11-KT fraction (30%) may have occurred. Fractions were collected in 13 X 100 mm borosilicate culture tubes and dried as above. These were reconstituted in PBS-gel assay buffer, tightly capped, and placed either in a 40° C water bath for approximately two hours or in a refrigerator at 4°C overnight.

Radioimmunoassay methods and cross-reactivities of antisera for testosterone, 11-ketotestosterone, estradiol-17β, and cortisol have been described previously (Singh *et al.*, 1988; Robertson and Thomas, 1988). Column fractions were reconstituted in 225μl and two aliquots (50 and 100 μl) were run in each assay as a check on parallelism, while 50μl was used to estimate extraction efficiency. Recovery of known amounts of cold steroids (50-250 pg/tube) added to control bloods ranged from 84.1-127.7% (x=105.3%). RIA methods for androstenedione were identical to those above. Androstenedione antiserum was purchased from Endocrine Sciences (AN6-22; Tarzana, CA). Cross-reactivities of this antiserum (supplied by Endocrine Sciences) are shown in Table 2. Cortisol was assayed as follows. Twenty-five microliters of cortisol tracer (~3400 cpm) and 25μl of antiserum (sufficient to give ~50% binding in the absence of cold steroid) were added to both samples and standards. Three aliquots per sample (100, 50, 25μl) were run in each assay both as a check on parallelism and because of the large range of values typically encountered with this steroid in plasma. Following overnight incubation at 4ºC, bound and free steroid were separated with dextran-coated charcoal.

Standard curve sensitivity was considered to be the amount of cold steroid necessary to displace 5% of bound tracer. Table 3 illustrates the sensitivity and intra- and interassay variability for the various steroid RIAs. Intra- and interassay variability were determined using aliquots of the plasma pool gathered from captive females (described above). Samples were assayed in a relatively small number of assays and interassay variability was high in some cases (Table 3).

Table 2. Cross-reactivities of Androstenedione antiserum.

Steroid	% cross reaction
1,4-androstadiene-3,17-dione	40.0
5α-androstan-3,17-dione	35.0
5β-androstan-3,17-dione	35.0
adrenosterone	4.0
epiandrosterone	4.0
androsterone	3.5
5α-androstan-3,11,17-trione	2.5
testosterone	2.0
5β-androstan-3β-ol-17-one	0.6
5β-androstan-3,11,17-trione	0.5
11-hydroxyandrosterone	0.2
5β-androstan-3α,6α-diol-17-one	0.16
5β-androstan-3α,17β-diol	<0.1
5β-androstan-3β,17β-diol	<0.1
5β-androstan-17α-ol-3-one	<0.1
5α-androstan-3β,17β-diol	<0.1
progesterone	<0.1
17-hydroxyprogesterone	<0.1

In order to guard against the possibility that inter-assay variability would produce spurious differences between treatments, approximately equal numbers of samples from each treatment (5d, 10d, 20d, males, and females) were run in each assay. The influence of variability between assays should therefore be to make comparisons and interpretation conservative; obscuring real differences rather than indicating spurious ones. Results were unusable from some assays due to poor assay performance, and samples sizes for group comparisons therefore differ for the different steroids measured.

Table 3. Mean assay recoveries (%) and sensitivities (standard curve sensitivities are in pg/tube, assay sensitivities are in ng/ml).

Hormone	n	Recoveries	Sensitivity Curve	Sensitivity Assay	Intra-assay variation	Inter-assay variation
Androstenedione	3	80.3	2.4	0.130	2.8	20.2
Testosterone	2	73.1	2.7	0.170	8.4	5.6
11-ketotestosterone	3	73.7	2.3	0.140	11.1	13.0
Estradiol-17β	2	54.8	3.5	0.290	8.9	13.0
Cortisol	2	64.1	3.0	0.650	4.9	4.3

Histology

Gonads were dissected whole from experimental males at the termination of experiments and preserved in Bouin's fluid for 36-48 hours before being transferred to 50% ethanol. Further dehydration was performed in an ethanol-butanol series and infiltration and embedding was in Paraplast. Eight micron sections were taken on a standard microtome and stained with hematoxylin and eosin. Gonads from all experimental fish were histologically examined for confirmation that individuals were in the stage of gonadal transition discussed in the introduction and described by Godwin (in prep.).

Statistical Analyses

All statistical analyses were performed using the PC-SAS system (SAS Institute, Cary, N.C.). Where significant differences among treatment groups were indicated by analysis of variance, the Tukey-Kramer multiple comparison procedure was employed to localize these differences. Raw data for levels of androstenedione, testosterone, 11-ketotestosterone, and estradiol-17β were $\log_{10}$ transformed to homogenize variances among treatment groups. Non-

transformed means and standard errors are shown in figures. Spearman's rank correlation coefficient (r_s) was used to explore associations between variables within individuals. Unless otherwise noted, a significant result refers to rejection of the null hypothesis of no difference at an α level of 0.05 and error bars represent 1 standard error of the mean.

Results

Gonadal Steroids

Significant differences in levels of the three androgens measured were found among males, females, and the three points in sex change (p<0.001; Figure 1a-c, Table 4). Plasma concentrations of Ad were significantly higher in females than males, while T levels were not, probably due to the high variability in female T levels (0.1>p>0.05). 11-KT exhibited the opposite pattern. Males had significantly higher levels than females (p<0.01). Levels of 11-KT were considerably lower than those of either T or Ad in both sexes, especially females.

The onset of sex change was accompanied by a general trend of decreasing androgen levels. The only significant difference detected in comparisons of sex-changing individuals with unmanipulated males was seen in T concentrations in ten day intersexuals. Testosterone levels were below assay detection limits in four of five individuals for which measurements were obtained at ten days into sex change. Both T and Ad levels were significantly lower in intersexual animals than in mature females (p<0.01 for both Ad and T). 11-KT levels showed a decreasing trend with time in sex change and the assumption of female function, but the only significant difference found was between males and females (p<0.01).

Mature females had levels of E_2 approximately seven times higher than males and both five and ten day intersexual individuals (*a posteriori* comparisons: p<0.01; Figure 1d, Table 3). There was no evidence of an increase in E2 levels until 20 days after the onset of sex change. Twenty day intersexuals had significantly higher E_2 levels than either males or five day intersexuals, and significantly lower levels than females (p<0.01). Considerable variation was seen

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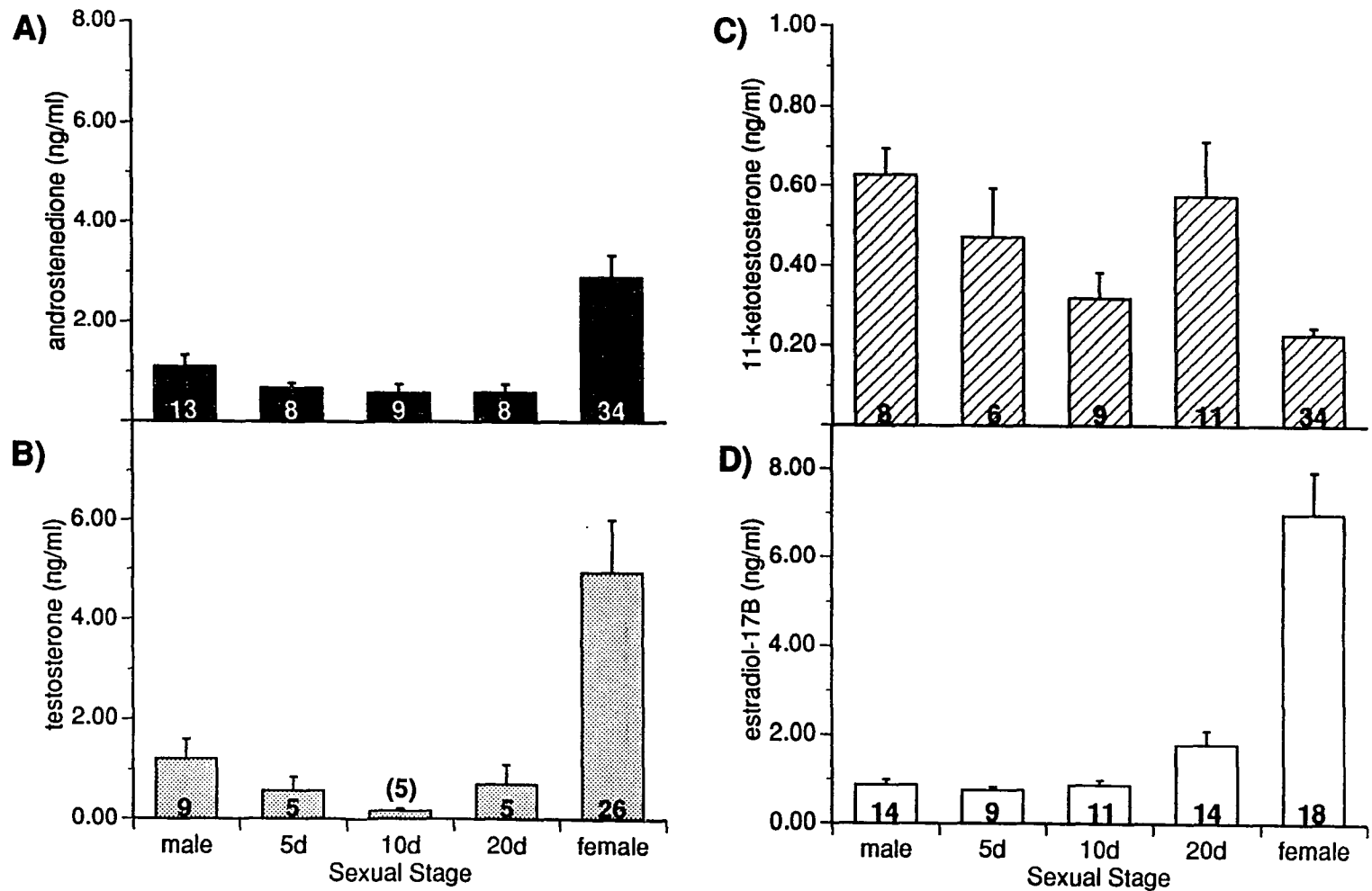
Figure 6. Plasma steroid levels in *Amphiprion melanopus* males, females, and intersexes at 5, 10, and 20 days after female removal. A: androstenedione, B: testosterone, C: 11-ketotestosterone, D: Estradiol-17b (numbers in bars are samples sizes, error bars=1 SEM).

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in E_2 levels in the twenty day treatment. Rather than a moderate increase being seen in all individuals, it appeared that some had E_2 elevations above male levels while others did not. Seven of the 20d fish had E_2 levels which were above the 99% confidence limit for males while six others had levels within these limits. This suggests that E_2 production had increased above male levels in some individuals by 20 days. There was no evidence of such an increase at ten days.

Table 4. Results of ANOVA and Tukey's HSD multiple comparison tests.

Steroid	ANOVA test statistic (df)	p	significant multiple comparisons
Androstenedione	15.15 (4, 67)	<0.001	F > M (p<0.05) F > 5, 10, 20d (p<0.01)
Testosterone	15.40 (4, 45)	<0.001	F = M (0.05<p<0.10) F > 5, 10, 20d (p<0.01) M > 10d (p<0.05)
11-ketotestosterone	6.48 (4, 63)	<0.002	M > 10d, F (p<0.01)
Estradiol-17β	42.46 (4, 60)	<0.001	F > M, 5, 10, 20d (p<0.01) 20d > M (p<0.05) 20d > 5d (p<0.01)
Cortisol	11.78 (4, 60)	<0.001	20d > M, 5, 10d, F (p<0.01)

Significant correlations were not observed between the levels of Ad, T, and E_2 in intersexual fish or between any of the gonadal steroids and time elapsed from initial approach to sampling. Within individuals, Ad levels were correlated with T in males (Ad&T: $r_s=0.767$, p<0.05) and both T and E_2 in females (Ad&T: $r_s=0.950$, p<0.001; Ad&E_2: $r_s=0.540$, p<0.05). Testosterone levels were correlated with E_2 levels in females ($r_s=0.806$, p<0.005), but not males ($r_s=-0.200$, p>0.5).

Gonadal Steroid Ratios

Ratios of E2 to both Ad and T showed increases after the onset of sex change, although these ratios were not different between males and females (Figure 7a). The difference between males and females in the ratio of E_2/11-KT increased twenty-fold, from 1.64 to 29.96 (Fig. 7a). A steady increase in this ratio after the onset of sex change was suggested, but these differences were not significant. Since an androgen receptor might not distinguish completely between steroids, a ratio of E_2 to androgens measured was generated from group means (Fig. 7b). This ratio showed a steady increase with progress from the male to ten days, at which it reached approximately female levels. This was coincident initially with the decrease in mean Ad and T levels and then with decreased 11-KT levels in females.

Cortisol

Plasma cortisol exhibited a trend towards increased levels with increasing elapsed time from initial approach to sampling for both males (Spearman $r_s=0.508$, p=0.064) and females ($r_s=0.422$, p=0.081), but not intersexual fish at any stage (p>0.49 for all three treatments) (Fig. 8).

Cortisol levels among treatments exhibited an unusual pattern (Fig. 9, Table 4). Males and females did not differ, but an increase over male levels after the onset of sex change was indicated. This increase was not significant at five or ten days after female removal. Twenty days after female removal, however, mean cortisol levels were approximately three times higher than those observed in either males or females and double those observed in five and ten day intersexuals (20d intersexuals vs. all other stages: $p<0.01$). This is a conservative comparison as the mean time from initial approach to sampling was longer for females (632 s) and males (641 s) than for intersexual fish (5d=414s; 10d=303s; 20d=372s). Even in samples for which the elapsed time was relatively short in intersexual fish (<300 s), high levels of cortisol were observed (Fig. 8), suggesting that these levels were elevated prior to capture and handling stress.

A)

B)
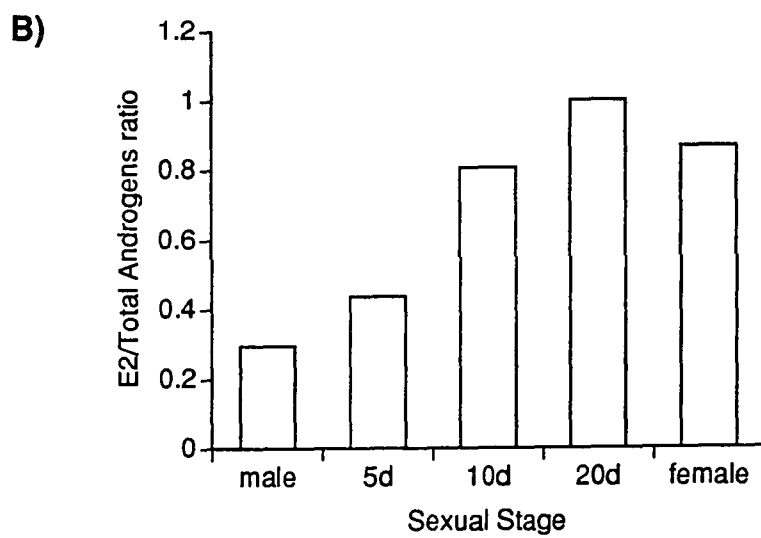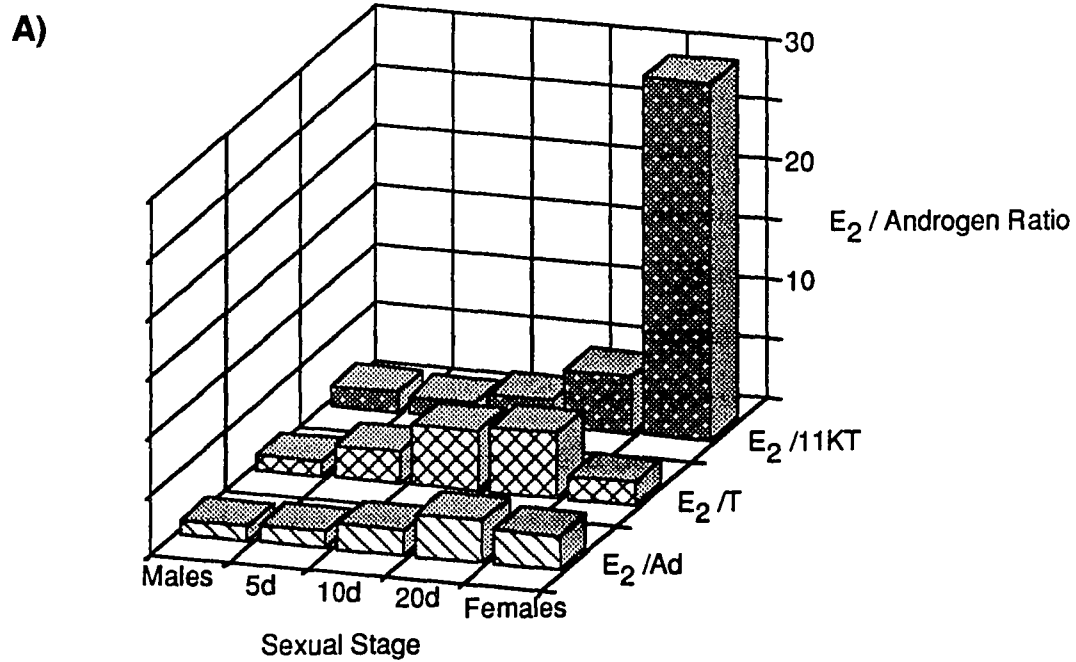
Figure 7. Ratio of E_2 to a) individual androgens and b) total androgens.
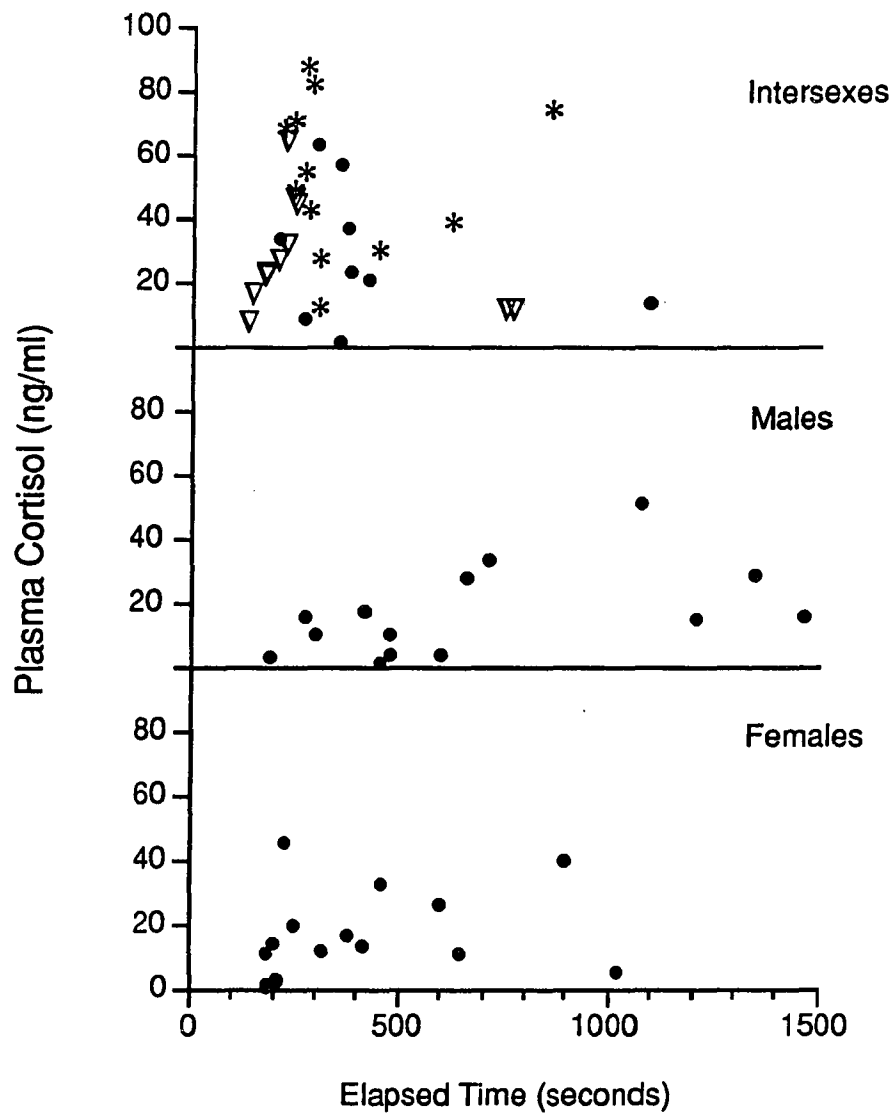
Figure 8. Plasma cortisol levels in male, female, and intersexes with time
elapsed from approach to sampling (intersexes: 5d - closed circles,
10d - open inverted triangles, 20d - asterisks).

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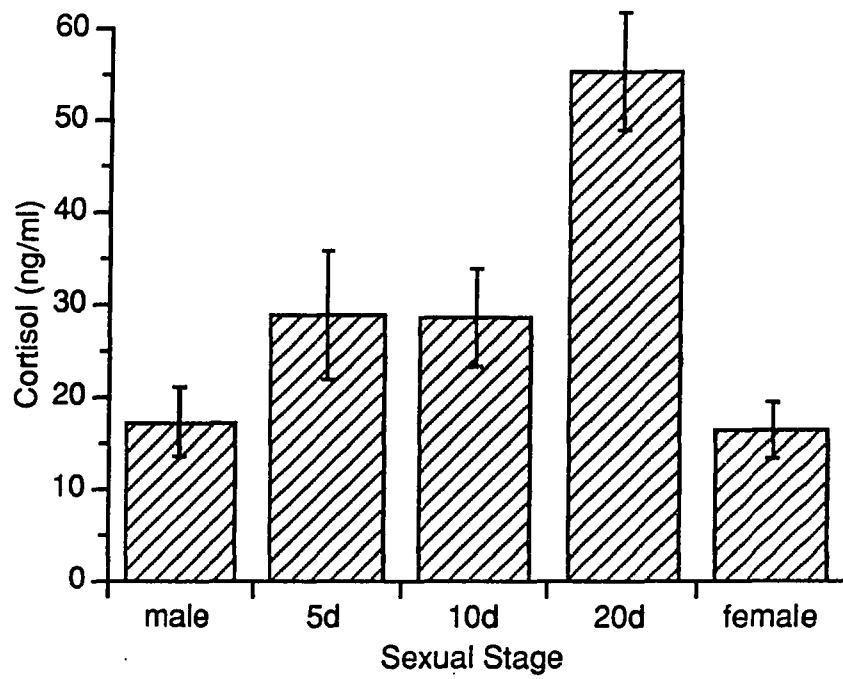
Figure 9. Plasma cortisol levels at different sexual stages (error bars = 1SEM).

Discussion

The physiology of sex change in fishes has been the subject of many studies, but only recently has information on blood levels of steroid hormones for fish undergoing sex change been obtained. Information on circulating gonadal steroids is now available for six species (including the present study). The pattern of differences in blood levels of gonadal steroids found between male and female *A. melanopus* is similar to that described for three protogynous species (*Thalassoma duperrey*: Nakamura *et al.*, 1989; *Sparisoma viride*: Cardwell and Liley, 1991; *Centropristes striatus*: Cochran and Grier, 1991), but different from that of two others (the protandrous *Rhabdosargus sarba* and protogynous *Monopterus albus*, Yeung and Chan, 1987a,b). More variation is seen between species in steroid patterns during the sex change process. However, it is difficult to separate the effects of differing methodologies and life history patterns of the species concerned (e.g., seasonal reproduction) from fundamental physiological differences relevant to the mechanism of sex change.

Gonadal Steroids

The relative importance of different androgens, especially T and 11KT, in teleost male reproductive function is unclear and probably differs between species. Roles of androgens in both sexual differentiation and spermatogenic and spermiogenic processes have been demonstrated (Fostier, *et al.*, 1983; Billard *et al.*, 1990). Recent evidence from *Fundulus heteroclitus* indicates differing, but important, functions for T and 11KT. *In vitro*, T stimulated the development of spermatogonia into spermatocytes, but neither 11KT or 11β-hydroxytestosterone [11OHT] did (Cochran, 1992). A role for 11KT in spermiation was suggested by a synchronization of peak plasma levels with spawning (Cochran, 1987). Various

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androgens have been used to successfully manipulate sex of gonochores in early development (reviewed in Hunter and Donaldson, 1983) and 11KT induced precocious sex change in one protogynous species (*S. viride*: Cardwell and Liley, 1991a) and changes to male-characteristic Gonadotropin-releasing-hormone (GnRH) cell numbers in another (*Thalassoma bifasciatum*: Grober and Bass, 1991a).

An hypothesis proposing direct or indirect causal influences of androgens in gonadal sex change would be supported by increases in blood levels at the earliest stages of male differentiation in protogynous change and decreases with loss of male function in protandrous change. Increasing androgen levels occurring with proliferation of spermatogenic tissue in protogynous change have been described in three species (*T. duperrey*: Nakamura *et al.*, 1989;*S. viride*: Cardwell and Liley, 1991a; *C. striatus*: Cochran and Grier, 1991), but not *M. albus*: (Yeung and Chan, 1987a). Decreases in androgens were not seen in the protandrous *R. sarba* during sex change (Yeung and Chan, 1987b).

Both the higher levels of 11KT observed in male *A. melanopus* and the trend toward initial decreases in all three measured androgens (significant for T) during sex change suggest influences of these steroids on male function in this species, supporting the above hypothesis. Female teleosts typically have lower 11KT levels than males. This pattern is also seen in the sex-changing *T. duperrey*, *S. viride*, and *C. striatus*, but not *M. albus* or *R. sarba*, which show no sex differences. A primarily spermatogenic or spermiogenic function for 11KT would be consistent with the higher levels observed in male *A. melanopus*.

In contrast to the pattern seen in 11KT, females exhibit higher T levels than males in a number of teleosts (e.g., Burke and Leatherland, 1984; Prat *et al.*, 1990), including the protogynous *T. duperrey*. Unlike 11KT, T is converted to E_2 via

aromatization and high levels of T in females may simply reflect steroidogenic processes leading to E_2 production or "leftover" precursor when aromatization declines (Campbell et al., 1976). The strong correlation between T and E_2 levels in individual female *A. melanopus* supports this interpretation and is similar to findings in channel catfish (MacKenzie *et al.*, 1989). The complete lack of spermatogenic tissue in female *A. melanopus* despite higher T levels does not necessarily argue against a role for this steroid in spermatogenesis. A spermatogenic potentiality may be lost in the germ cells following sex change.

T and 11KT drop from male levels by the ten day point of sex change in *A. melanopus*. Nearly all spermatogenic tissue in the gonad has matured, early spermatogenic stages are rare or absent, and oogonial proliferation has begun in some individuals (Godwin, Chapter II, in prep.). Ten days later (20d) this peripheral area of the gonad is filled with primary oocytes and spermatogenic tissue is rare. These gonadal changes suggest that an alteration in the pathway of differentiation occurs in the gonad before or around the ten day point. Callard (1992) has found that steroid involvement in shark (*Squalus acanthias*) and salamander (*Necturus maculosus*) spermatogenesis is most important in the early spermatogenic stages. A directive influence of androgens on differentiation of early spermatogenic stages in *A. melanopus* would be consistent with the observed coincident decrease in androgens and cessation of recruitment of germ cells into spermatogenesis which occurs in the first ten days of sex change.

Little information is available on levels or functions of Ad in either male or female fishes. Yeung and Chan (1987a,b) found that Ad was higher in females than males of *Monopterus albus* at all stages and in *Rhabdosargus sarba* during some stages of the reproductive cycle. As with T, Ad is a biosynthetic

intermediate in steroidogenesis in at least some fishes (Fostier *et al.*, 1983). The correlation of Ad levels with T in male *A. melanopus* and both T and E_2 in females suggests that plasma concentrations of this steroid are coupled with synthesis of steroids further along this biosynthetic pathway. Yeung and Chan (1987a) found increased levels of Ad in *M. albus* both *in vitro* and in the blood during sex change, and suggested this steroid might be involved in sex change. The opposite pattern was seen in plasma levels in *A. melanopus*, suggesting no special role for Ad during sex change.

The function of 11β-hydroxytestosterone (11OHT) in male reproductive function in teleosts is not well understood. This androgen was not measured in *A. melanopus*, but may have been quantitatively important. 11OHT was originally proposed to be the most important androgen in hermaphroditic teleosts by Idler and coworkers (but see Kime and Hyder, 1983) and is found in higher levels in males of the single protandrous species where it has been measured, *R. sarba* (Yeung and Chan, 1987b; Chan and Yeung, 1989). 11OHT was also the predominant metabolite from T *in vitro* in *R. sarba* (Yeung and Chan, 1985), males of two protogynous species (Reinboth and Becker, 1984; Hourigan *et al.*, 1991), and males and juveniles of *Amphiprion frenatus* (a close relative of *A. melanopus*: Latz *et al.*, 1991). In female *A. frenatus*, 11KT was quantitatively most important *in vitro*. Interestingly, no marked decrease in 11β-hydroxylase activity was observed during sex change in *A. frenatus* despite the fact that testicular tissue was greatly reduced in the gonad (11β-hydroxylase activity did drop dramatically in females). This finding is supported by the relatively high 11KT levels seen in 20 day intersexual *A. melanopus*, which also had very little spermatogenic tissue. The *in vitro* results from *A. frenatus*, with

greater 11OHT production by males and juveniles, suggest plasma levels of 11OHT might show even greater differences than 11KT between sexual stages in *A. melanopus*.

It is relevant here to note 11OHT is quantitatively the most important androgen in the plasma of male *F. heteroclitus*, but measurements taken over the reproductive cycle did not suggest a function for this steroid (in contrast to T and 11KT in this species: Cochran, 1987, 1992). 11OHT could be a precursor for 11KT in both *A. melanopus* and *F. heteroclitus* as suggested by Hourigan and coworkers (1991) for the protogynous *T. duperrey*. Extragonadal conversion of 11-oxygenated androgens has been demonstrated for a number of teleost groups (Mayer *et al.*, 1990), although the specific conversion of 11OHT to 11KT has not.

Higher levels of E_2 in female than male *A. melanopus* are in agreement with the known functions of this steroid in other fishes. As above with 11KT, *T. duperrey*, *S. viride*, and *C. striatus* showed a similar pattern of sex differences in E_2 levels to *A. melanopus*, while no sex differences were found in *M. albus* or *R. sarba*. The generally-accepted primary function of E_2 in teleosts is the stimulation of vitellogenic processes in the gonads and hepatic tissue (Ng and Idler, 1983; Fostier *et al.*, 1983). Other proposed functions include roles in sexual differentiation and ovarian recrudescence, although the evidence is conflicting and interpretation is complicated by potential feedback effects on the pituitary (Khoo, 1975; Fostier *et al.*, 1983). In some seasonally-breeding species, ovarian recrudescence precedes measureable increases in circulating E_2 (e.g., Pankhurst and Conroy, 1987).

As discussed above for androgens, an hypothesized role for E_2 in ovarian differentiation during protandrous sex change would be supported by increases

in plasma levels prior to or coincident with the first visible gonadal changes. Patterns of plasma E_2 during sex change in *A. melanopus* did not implicate this steroid in the initial events of gonadal change. The first signs of gonadal change were seen at ten days, but E_2 did not show an elevation over male levels before the 20 day treatment. The gonad has become an immature ovary by this stage. This result agrees with findings that steroidogenesis begins only after the onset of ovarian differentiation in other fishes (Van den Hurk *et al.*, 1982; Rothbard *et al.*, 1987; but see Feist *et al.*, 1990). Caveats must be stated here, however. It is possible that differences would have been found had sampling been performed differently with respect to time of day. This seems improbable given that samples were taken over the entire daylight period for each of the treatment groups, and data inspection revealed no obvious diurnal patterns. A second possibility is that plasma concentrations do not reflect effective concentrations in gonadal tissue. Measurement of both gonadal and circulating concentrations of gonadal steroids in diverse animals have shown that gonadal concentrations may be many times higher (Idler *et al.*, 1971; Garnier and Joly, 1991). Finally, an increase in estrogen receptor levels early in sex change could increase the effect of E_2 without a change in plasma concentration.

A role for estrogens in gamete duct development in teleosts is well established (Fostier *et al.*, 1983). The ovarian lumen of functional female *A. melanopus* forms by a progressive rostral-to-caudal invagination of a band of ovarian tissue, enclosing a luminal space and eventually establishing continuity with the gonoduct during sex change (see Godwin, Chapter II & in prep. for details). Lumen formation is completed late in the sex change process (30-45+ days). In agreement with observations for gonochoristic species then, this part of

sexual differentiation occurs after the initial increase in E_2 concentrations in
A. melanopus and may be influenced by them.

While changes in E_2 were not seen before or during visible gonadal
changes, changes in the ratios of E_2 to individual androgens and total androgens
did occur after the onset of sex change (Fig. 2a,b). The steady increase in these
ratios could exercise influences on germ cells, or act through feedback effects
further up the hypothalamic-pituitary-gonadal axis. Bogart (1987) proposed a
parsimonious hypothesis for diverse sex determination processes in vertebrates
based on the action of aromatase and control of androgen/estrogen ratios in
developing gonads. Support for this hypothesis was recently provided by the
complete masculinization of phenotype in chickens achieved through
administration of aromatase inhibitors in early development (Elbrecht and Smith,
1992). Aromatase has been detected in early development in fishes (van den
Hurk *et al.*, 1982), but little is known of its patterns in sex changers.

Cortisol

The primary function of cortisol in fishes appears to be in the stress
response (Mazeaud *et al.*, 1977; Thomas, 1990). Consistent differences have been
found over the course of semilunar and seasonal reproductive cycles (Bradford
and Taylor, 1987; Wingfield and Grimm, 1977; Lamba *et al.*, 1983), between
immature or regressed and mature fish (Peter *et al.*, 1978) and dominant and
subordinate individuals in dominance heirarchies (Scott and Currie, 1980;
Hannes, 1985). Direct inhibition of gonadal steroidogenesis *in vitro* has been
shown in both mammals and fish (Rivier and Rivest, 1991; Safford and Thomas,
in prep.; Carragher and Sumpter, 1990). Cortisol measurements were
undertaken in this study primarily to examine the influence of social interactions

and possible links between social stress and gonadal function. The lack of apparent differences in measured levels or response to capture stress between males and females suggests that cortisol is not important in the prevention of sex change in males or linked with their subordinate status, although further work is necessary here. Measurements from subordinate, non-breeding individuals would be useful in this regard. During sex change, important alterations in both social interactions and gonadal morphogenesis occur in *A. melanopus*. However, while aggressive interactions increase immediately after female removal and peak between one and five days (Godwin, chapter IV, in prep.), the highest observed levels of cortisol were seen at 20 days. This argues against a strict link to social interactions. Wingfield and Grimm (1977) and others have suggested that elevated cortisol levels in seasonally-reproducing species mobilize energy reserves. The energetic cost of gonadal transformation is difficult to estimate, but might be expected to be greatest among the treatment groups sampled here at 20 days, when previtellogenic oocytes are forming the bulk of the gonad. Unfortunately, there is no information on cortisol in other sex-changing fishes for comparison.

Recently, a primacy of female sexual development similar to that of mammals has been suggested for teleosts (Francis, 1992; Shapiro, 1992). Attempting to reconcile the occurrence of protandry with this general pattern, Shapiro convincingly argued that male function in *Amphiprion* species and protandrous sparids is temporarily "superimposed" on an underlying female developmental pathway by a masculinizing influence. In support of this hypothesis, sexual development in *Amphiprion* species initially appears to proceed along a female pathway. Assumption of male gonadal function appears

to be facultative in at least two species of anemonefishes (*A. frenatus*:
Stahlschmidt and Reinboth, 1988; *A. clarkii*: Hattori and Yanagisawa, 1991).
Blood concentrations of androgens were not measured in non-breeding
individuals in this study, but the decrease in these hormones with the loss of
male function is consistent with Shapiro's hypothesis. Non-breeding juveniles
would be expected to have lower androgen levels than functional males. During
sex change, perhaps falling levels of these putative male sex-inducers allow
development of gonial tissue to instead resume a "default" path, that
of oogenesis.

The evidence presented here for a role of steroid hormones in sex change
is strictly correlative: experimental manipulations are the next critical step.
Because of the ease of working with anemonefishes in both the field and
laboratory and the precise control of sex change possible with social induction of
the process, these fishes provide an excellent system to rigorously test
hypotheses about the physiology of sex change. A general consensus on
physiological mechanisms of sex change in the teleostei may be difficult to reach
because of the diversity and long evolutionary history of this group of fishes.
The general agreement between results for *T. duperrey* and *S. viride* and those
described here for *A. melanopus*, and lack of agreement with Yeung and Chan's
work with *R. sarba* and *M. albus* may simply reflect the closer phylogenetic
relationship among the first group. Labrids (*T. duperrey*), scarids (*S. viride*), and
pomacentrids (*A. melanopus*) are considered part of a monophyletic group classed
together in the suborder Labroidei within the Perciformes (Kaufman and Liem,
1982). The Sparidae (*R. sarba*) is not placed in the Labroidei and *M. albus* belongs
to a different order, the Symbranchiformes. At least three families of fishes

display the three basic reproductive patterns of gonochorism, protandry, and protogyny: the Sparidae (Buxton and Garratt, 1990), Muraenidae (which also contains simultaneous hermaphrodites: Fishelson, 1992), and Pomacentridae (Warner, 1984). Comparative study of closely-related fishes within these groups exhibiting divergent reproductive patterns should prove valuable.

CHAPTER IV

BEHAVIORAL ASPECTS OF PROTANDROUS SEX CHANGE AND ENDOCRINE CORRELATES

Introduction

Steroid hormones play an important role in the control of behavior in many vertebrates. Androgens in particular are known to be organizers and/or activators of aggressive and reproductive behaviors. Examples of critical roles for these hormones in development or expression of these behaviors are numerous and come from all vertebrate groups (Goy and McEwen, 1980; Kelley, 1988; Moore, 1991; Arnold, 1990). However, the study of atypical model species demonstrates that a strict association between steroid hormones and aggressive or reproductive behavior is not universal (Crews and Moore, 1986).

One difficulty with interpretation of possible links between androgen levels and behavior in males of species with typical sex-role patterns is that potential roles of these hormones in producing "male" behavior are completely confounded with the androgen requirements of testicular function. Hence, sexual dimorphisms in androgens in such behaviorally-dimorphic species do not necessarily implicate these steroids in behavioral control. Species with a temporal dissociation between gametogenesis and breeding demonstrate that male reproductive behavior need not be androgen dependent (Crews and Moore, 1986). Two other approaches to this problem have included comparisons of phenotypes i) within sexes (alternative male phenotypes), so that while behavior differs, the androgen requirements for gonadal function should be similar, and ii) between sexes where androgen requirements for gonadal function differ, but behavioral sex roles are reversed and "male-like" behavior is shown by females

(polyandrous species, ie.-change the gonad, but keep the "male" behavior). Androgens are associated with the expression of alternative male phenotypes (reviewed in Moore, 1991), but androgen-level reversals do not account for the behavioral sex-role reversals observed in polyandrous birds (e.g., Gratto-Trevor *et al.*, 1990; but see Oring *et al.*, 1986 for evidence of reversals in prolactin).

Two observations emerge from these studies. The first is the primacy of gonadal function. Where gonadal and behavioral requirements for steroidal modulation differ, gonadal requirements take precedence. Kelley (1988) postulated that gametogenic steroidal requirements constrain alteration of steroid levels for behavioral control (see also Moore, 1991). The first observation leads to the second: the utilization of gonadal steroids as proximate stimulators of behavior is opportunistic and flexible in an evolutionary sense (Crews and Moore, 1986). Where a steroid is elevated at an appropriate time to cue aggressive or reproductive behavior, due to gametogenic processes, it may do so. If a behavior is dissociated from gonadal processes, either temporally or through sex-role reversals, no steroid dependence is likely to occur.

Sex change in fishes provides a useful natural experiment through which the hormonal control of behavior may be examined. Individuals can be examined in different behavioral phenotypes (sex roles) and during transitions between these phenotypes. Behavioral studies of sex-changing fish have concentrated on protogynous (female-first) coral-reef species (Fishelson, 1970; Robertson, 1972; Warner *et al.*, 1975; Shapiro, 1981a,b; Ross *et al.*, 1983). Behavior of males often differs strikingly from that of females in these species, particularly in aspects of territorial aggression and courtship. However, from an endocrinological perspective, behavioral transitions accompanying sex change

are confounded with transformation of the gonad from an ovary to a testis in these species. Both the behavioral and gonadal transitions predict similar changes in gonadal steroids. Endocrine data on sex-changing species are limited, but steroid correlates of gonadal and behavioral function have been teased apart through examination of alternative male phenotypes in two diandric protogynous labroids (wrasses and parrotfish) which exhibit androgen differences between male morphs (Cardwell and Liley, 1991a,b; Hourigan *et al.*, 1991). The colorful and territorial terminal-phase males of both species exhibit higher levels of the potent teleost androgen 11-ketotestosterone either in gonadal steroidogenesis (*Thalasssoma duperrey*, Hourigan *et al.*, 1991) or circulating levels in plasma (*Sparisoma viride*, Cardwell and Liley, 1991a,b).

Anemonefishes are protandrous coral-reef fishes which provide a useful system for addressing the questions discussed above. The advantages of these fishes for study of the links between behavioral expression, gonadal function, and endocrine physiology derive from a reversal of sex roles with females as the behaviorally-dominant sex, and the change of sex which allows observations of transitions from one phenotype to the other within individuals as well as the transition period itself.

The biology of anemonefishes is well studied because of their unusual symbiotic association with large tropical sea anemones (Allen, 1972; Dunn, 1981). In all species examined, females are the behaviorally-dominant and most aggressive members of monogamously-mating pairs and of the social groups which occupy and defend as their territory various species of anemones. Male adult pair members undergo protandrous sex change upon disappearance or removal of their female mates and the largest resident non-breeding individual,

the α-subadult, assumes the male breeding position. Sex change has been studied in various species of anemonefishes (Fricke and Fricke, 1977; Fricke, 1979, 1983; Moyer and Nakazono, 1978; Ochi, 1989; Hattori, 1991; Hattori and Yanagisawa, 1991) Protandry was first described in *A. melanopus*, the subject of this study, by Ross (1978a). *Amphiprion melanopus* provides several advantages as a model species for the study of sex change in the field. It is relatively common, found in shallow water, easily captured, and extremely site-attached. Detailed analysis of the gonadal histology of the sex change process and its endocrine correlates in this species has been provided by Godwin (Chapter II, III) and Godwin and Thomas (in prep.).

This paper describes behavioral differences between male and female *A. melanopus* and the nature and time course of behavioral changes occurring with sex change with samples taken before, during and after critical events of gonadal change. The general hypothesis that these behavioral differences between the sexes and changes with sex change are related to plasma steroid hormone levels is examined both on an individual level and in overall pattern.

Materials and Methods

Study location and habitat characteristics

All field work reported on here was performed from the Christensen Research Institute (CRI) near Madang, Papua New Guinea (5°11' S, 145°50' E) during June-August, 1989 and July-August, 1991. Groups of *A. melanopus* were located in the field on small coral patch reefs and along the outer barrier reef in 1-8 m water depth within 2 km of CRI.

Handling, capture methods, and stimulation of sex change

Fish were captured underwater during daylight hours (0700-1800h) either snorkeling or with SCUBA using handnets and quinaldine anesthetic (2-methyl quinoline). Sex change was induced in male *A. melanopus* through removal of their female mates. Both members of a pair were captured and transferred to a small boat. Sex can be reliably determined in the Pomacentridae by examination of the sexually dimorphic genital papilla (Thresher, 1984). Additionally, gentle pressure on the abdomen usually causes extrusion of either milt or eggs, verifying sexual identifications. Males were individually marked (fin-clipped), measured to the nearest mm (standard length), and released back on their anemones within 15 minutes. These males were later recaptured after being allowed to undergo sex change for varying periods. Females were maintained in aquaria with running seawater until the termination of experiments and released back onto anemones in the field. Care of demersal eggs is performed primarily by the male in this species and reproductive activity exhibits a semi-lunar cycle in Guam with spawning taking place around the quarter moons (Ross, 1978a). This also appeared to be the case with the study population in Papua New

Guinea. Female removals were performed in the week preceding quarter moons to avoid unpredictable effects of the presence of eggs on male behavior, the initiation of sex change, and steroid levels. Female removal was not performed if it was known that a pair currently had eggs. That no removals were performed while eggs were present cannot be stated with certainty. Eggs were not always easily visible, but their presence was usually apparent from egg care activity of males even when they were not visible (see Allen, 1972).

Behavioral measurements

Eleven behavior patterns were recognized. These are described and defined in a partial ethogram (Table 5). Ten-minute, focal-individual behavioral samples were taken in both 1989 and 1991. All observations were made during daylight hours. All behavioral frequencies are expressed as number of acts per 10 minute sample. The order of sampling on any given day was not consistently randomized and was determined to some extent by tidal conditions and the presence of more than one experimental group in a given location. The beginning of an observation session was determined by time of arrival at an experimental group. Two baseline samples were taken on separate days (1-11 days apart) for each male prior to female removal in both years. The means of these two baseline samples were then used for within-individual comparisons to measures of behavior taken after removal of the female.

Different sampling times and methods were used in 1989 than 1991. In 1989, post-female removal behavioral samples were taken at 10, 20, 30, and 45 days after female removal. This sampling design was chosen to cover a majority of the period from female removal to completion of sex change, which previous work by the author in Guam had shown to be longer than 30 days.

Table 5. *Amphiprion melanopus* behavior patterns.

Behavior	Description
approach	rapid, directed swimming movement which decreases the distance to a stationary or slow-moving conspecific or heterospecific
chase	rapid, directed swimming movement at an opponent fish which is also moving, usually in the same direction
flee	rapid swimming movement which increases the distance from a stationary or moving opponent fish
close face	focal individual places itself and is at least briefly stationary within 1/2 body length of recipient; the long axis of the body is oriented toward the recipient, head forward, and body is upright (not leaning)
close flank	same as close face, but side of body is oriented towards recipient; body is upright
raise dorsal	full erection of dorsal fin, and usually other fins, when in close proximity (3-10 cm) to another fish; observed during approaches, chases and lower level agonistic interactions
headshake	vigorous shaking of head with body relatively stationary, often seen when focal individual is recipient of attack
tail jerk	rapid, jerking movement of the tail from side to side; usually observed in small individuals when attacked by a larger fish
dorsal lean	a rotation about the longitudinal axis of the body as much as 45 degrees from vertical; observed when focal individual is recipient of attack, usually from a larger fish; direction of rotation is such that dorsal fin is directed at the attacker
substrate biting	focal individual bites, often repeatedly, at area of open substratum near the host anemone; body orientation is roughly perpendicular to the substratum. This behavior is often seen in males when they receive approaches from females.
bathe	focal individual places itself in contact with tentacles of the host anemone; often involves "burrowing" motions in which fish appears to get itself as far as possible into the anemone's tentacles
tentacle feed	focal individual bites an anemone tentacle near the base, detaches, and then consumes it
visit	the two largest fish in a group (pair members) are relatively motionless and bathe in a parallel orientation within 1/2 body length of one another.

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1989 experiments

♀ removal

baseline measures | 10d | 20d | 30d | 45d

Ten day experiments

Twenty day experiments

Thirty day experiments

Forty-five day experiments

sample sizes   20   17   14   8

1991 experiments

1d   5 d   10d   20d

Ten day experiments

Twenty day experiments

sample sizes   19   19   19   11
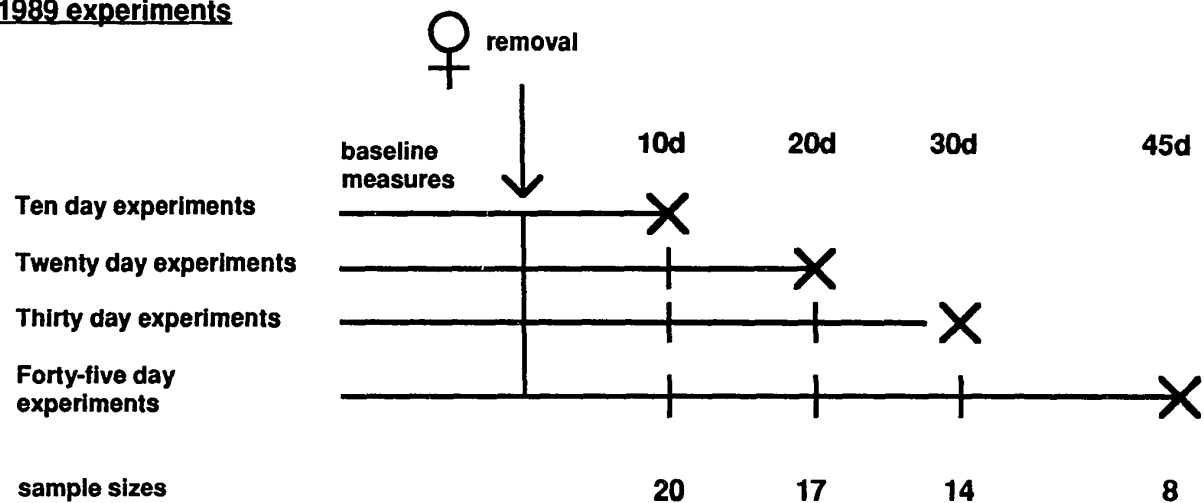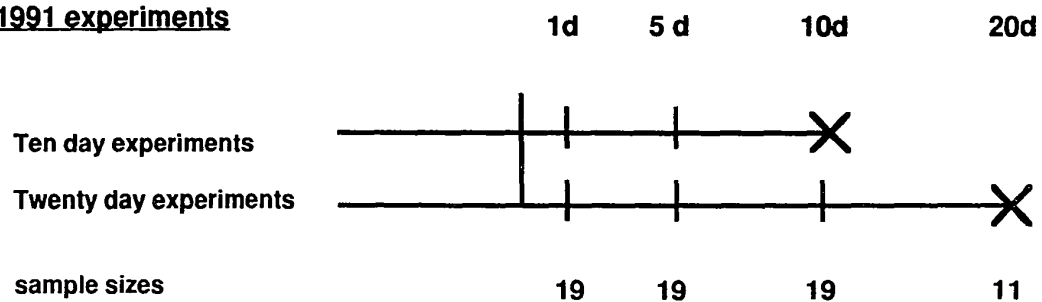
Figure 10. Sampling Design for 1989 and 1991 *Amphiprion melanopus* sex change studies.

Samples consisted of ten-minute videotaped sessions. Focal animals were videotaped from a distance of approximately 1.5m by a diver using either snorkeling or SCUBA equipment depending on water depth.

Continuous records of the behaviors described in Table 5 were generated from the tapes using the BEAST computerized event recording system (copyright Windward Technology, 1988). Further analyses of bout lengths and intervals for various behaviors using log-survivorship methods and behavioral sequencing using loglinear models (Colgan and Smith, 1978) and continous time Markov chain analysis (Haccou, 1987) were performed using the analytical facilities of the BEAST program. However, consistent differences in the temporal aspects and sequencing of different behavior patterns occurring with sex change were not found and these results are not included here.

In 1991, sampling focused on the initial period of sex change from one to 20 days. This is the period of critical gonadal and endocrine changes and results from the 1989 work suggested that dramatic behavioral changes might have been missed by not sampling until ten days after female removal. The primary behavioral change occurring in sex change as determined from the 1989 data was in the frequency of aggressive behavior. Consequently, sampling focused on the behavior "approach" in 1991. Post-female removal samples were taken at one, five, 10, and 20 days. The data recorded were the number of approaches given by the focal individual to either the female prior to her removal or the next largest individual in the group after the male (hereafter referred to as the "new male") following female removal, and other smaller fish which were resident in the focal individual's social group (hereafter referred to as "other" fish). "Total" approaches refers to all occurrences of the behavior "approach" recorded

for the focal individual in a given sample and is the sum of female/new male and other approaches. The numbers of approaches given by the female (pre-female removal) and the new male (post-female removal) in which the focal individual was the recipient were also noted. Data were recorded on underwater paper. This provided less total information than the complete record obtained for the 1989 samples, but allowed the initiators and recipients of approaches to be identified with more certainty than was possible from videotapes.

Analysis by paired and repeated-measures designs eliminated the need to correct for differences in the number of other fish resident in a given individual's social group for most comparisons. Where this correction was necessary, number of approaches was divided by the number of other fish resident in a group to provide the measure "approaches per resident fish". The behavior "visit" (Table 5) was recorded in most of the 1991 samples, but not for the 1989 samples. This behavior was not recorded in all the baseline samples for the sex-changing treatments in 1991, but was for the female/male paired observations and handling control experiment (see below).

Sample sizes decrease with increasing treatment length (time since female removal) in both the 1989 and 1991 work since some fish were captured at the end of each experimental period to obtain blood and gonad samples. The exceptions were the one and five day points in the 1991 work (Fig. 10). None of these fish were recaptured before ten days and samples sizes are therefore equal for the one, five and ten day comparisons.

A handling-control experiment was performed in 1991 to test the hypothesis that behavioral changes observed in the sex change experiments were due to capture and handling of the fish rather than female removal and/or the

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initiation of sex change. Males were captured, marked, measured and sexed as described above and held a standard 15 minutes from capture before being released back onto their anemones (this time was spent either in a handnet or bucket on the boat). Behavioral samples were identical to those described above for the 1991 sex-changing fish except that post-manipulation samples were only taken at one and five days. These were the sampling periods in which the most pronounced changes from baseline behavior were observed in sex-changing fish.

Simultaneous observations on the male and female members of 20 unmanipulated mated pairs of *A. melanopus* were performed in 1991 as part of an experiment on simulated territorial intrusion (results not reported here). This enabled pairwise comparisons of sex differences in aggressive behavior within pairs. The sampling method and behaviors recorded were the same as described above, but only a single behavioral sample was made for each pair.

Simulated Territorial Intrusion experiment

Ross (1978b) found that male and female *A. melanopus* show similar amounts of aggression toward a conspecific model intruder at the center of their territory, but that females showed much more aggression than males to an intruder presented at the periphery of the territory. An experiment simulating territorial intrusion was performed in this study to see if a change from the "male" pattern of territorial behavior to the "female" pattern could be detected in individuals undergoing sex change. A conspecific female (64 mm SL) in a clear glass "model bottle" (12 cm D X 20 cm H) was placed ~1m from the territorial center of a given group for seven minutes, removed for five minutes, then placed at the center of the territory for seven minutes. Attacks on the model bottle by a focal individual were counted for five of the seven minutes at each point after

allowing a two-minute "warming-up" period. Thresher (1978) found a rapid

increase in aggression occurred within the three minutes after a model bottle was

placed. Similar profiles of aggression increases with time were not examined in

this study and it cannot be stated whether such a "warming-up" takes place in

A. melanopus. An attack was defined as any approach or series of approaches to

within 1/2 body length of the bottle during which the focal individual did not

turn away from the bottle. These presentations were performed with the 45 day

experimental fish in 1989 before female removal (male baseline), and at 15, 30,

and 45 days following female removal. Model bottle presentation followed

behavioral sampling when these were performed on the same day. No attacks

on an empty bottle were observed. No other control was performed. In two

colonies, females moved in and replaced those that had been removed,

preventing sex change in experimental males. Results from these males are

also presented.

Blood Sampling and Steroid Determinations

Plasma levels of androstenedione (Ad), testosterone (T),

11-ketotestosterone (11-KT), estradiol-17β (E$_2$), and cortisol were determined in

sex-changing fish at five, 10, and 20 days after female removal in 1991. The 10

and 20 day fish were mainly those for which behavioral measures are reported

here. No behavioral data were obtained for the five day treatment individuals

used for steroid determinations. Blood samples were collected as quickly as

possible after the final behavioral sampling for a given sex-changing individual

with the intent of estimating plasma steroid levels present while the sampled

behavior was being performed. This allowed examination of the association of

behavior displayed by individuals with their plasma steroid levels. Fish were

captured as described above and transferred to a waiting boat. The time elapsed from when the diver came within 2 m of a focal individual's group to initiate capture until blood began to flow into the syringe after transfer to the boat was noted for each fish. Blood sampling methods, assay validations, and steroid separation and assay techniques are described in detail elsewhere (Chapter III and Godwin and Thomas, in prep.). The data on plasma steroid levels discussed here were presented in these other papers. Sample sizes for comparisons differ between the measured hormones because of assay problems (equipment failure, unacceptably low recoveries, experimenter error) that resulted in the loss of some samples.

Histology

The gonads of each sex-changing fish for which values are presented here were examined histologically to verify that sex change was indeed occurring and that the stage of gonadal transformation was that predicted from the amount of time the fish had allowed to change sex (see Chapter II). Gonads were dissected out, fixed in either Bouin's fluid or 10% buffered formalin, and worked up by standard histological methods. More detail is provided in Chapter II.

Statistics

All statistical analyses were performed either by hand calculation according to Zar (1984) or with PC-SAS (SAS Institute, Cary, NC). Comparisons of steroid levels were performed with one-way ANOVA and the Tukey-Kramer multiple comparison procedure. Raw data were $\log_{10}$ transformed to decrease heteroscedasticity for all steroids except cortisol.

For repeated measures ANOVA, type H covariance structure (Huynh and Feldt, 1970) was verified using the sphericity test provided by PC-SAS prior to interpretation of results from univariate analyses. Differences between correlated treatment measures have homogenous variances when data exhibit type H covariance, allowing the testing of mean square ratios from repeated measures ANOVA with the F distribution (Huynh and Feldt, 1970). Where the type H covariance assumption was violated, either the multivariate mode for calculating the F test statistic or adjustments to univariate numerator and denominator degrees of freedom as proposed by Huynh and Feldt (1976) and performed by PC-SAS were used. Huynh-Feldt adjusted probability values are noted in the text where used (e.g., "H-F adj. $p < 0.05$"). The multivariate test statistic does not rely on type H covariance structure, but is less powerful than the univariate equivalent at small sample sizes (Latour and Miniard, 1983). The F statistic could not be calculated in multivariate mode in some analyses due to insufficient error degrees of freedom.

Change in the frequency of single behaviors with time since female removal was the effect of interest in most of the analyses. These were indicated by "within-subjects" changes in the main effect "time". Following detection of a significant difference, treatment levels were compared with the "contrast" transformation in PC-SAS. Repeated measures multiple ANOVA was used to test for changes in overall behavioral profile in the 1989 data (10 behaviors X 2-5 times depending on experiment length). Both the main effect of "time" and the "time X behavior" interaction term were of interest in this analysis as indicators of changes in behavioral frequencies and the relative proportions of different behaviors in the time budget respectively. The between-subjects effect of

differences in frequencies of various behaviors was not of interest in the above analyses, but was in the simulated territorial intrusion experiment where the "time", "distance", and "time X distance" terms were all examined.

Spearman rank correlation coefficients were used to explore associations between variables.

Unless otherwise noted, a signficant difference refers to rejection of the null hypothesis of no difference at $\alpha = 0.05$ and error measures represent 1 standard error of the mean (S.E.M.).

Results

Unmanipulated male and female behavior

Simultaneous sampling of both male and female members of 20 unmanipulated pairs in 1991 showed that females were significantly more aggressive than males both when approaches on the other member of the adult pair and total approaches were considered, but not when approaches on only smaller fish were considered (paired-sample t tests, Fig. 11a). The pattern of sex differences is identical when approaches are corrected for the number of other fish resident in a colony (Fig. 11b). These comparisons consider aggression exhibited as a function of the number of potential targets of aggression, a measure of "opportunity". A dependence of approach frequency for males on "opportunity" was indicated by positive correlations of both total approaches and approaches on smaller fish with the total number of other fish resident in a colony (total approaches and number of resident fish: $r_s=0.340$, $p<0.05$; approaches on smaller fish: $r_s=0.489$, $p<0.002$, $n=40$). Neither measure of approaches was correlated with number of other fish for females or with body size (SL) for either males or females. The frequency of female approaches on males derived from single samples of pairs shown in Figure 11 (1.850 ± 0.75, range: 0-13, Table 6) agreed well with those derived from the means of baseline comparisons of female approaches directed at males prior to female removal in the sex-change experiments (2.141 ± 0.247, $n=32$, range: 0-9; this figure includes the handling control experiment baselines). There was an apparent difference between the groups for male approaches on females (unmanipulated pairs: mean=0.200 ± 0.090, baselines for sex changers: 1.321 ± 0.216), but this was not

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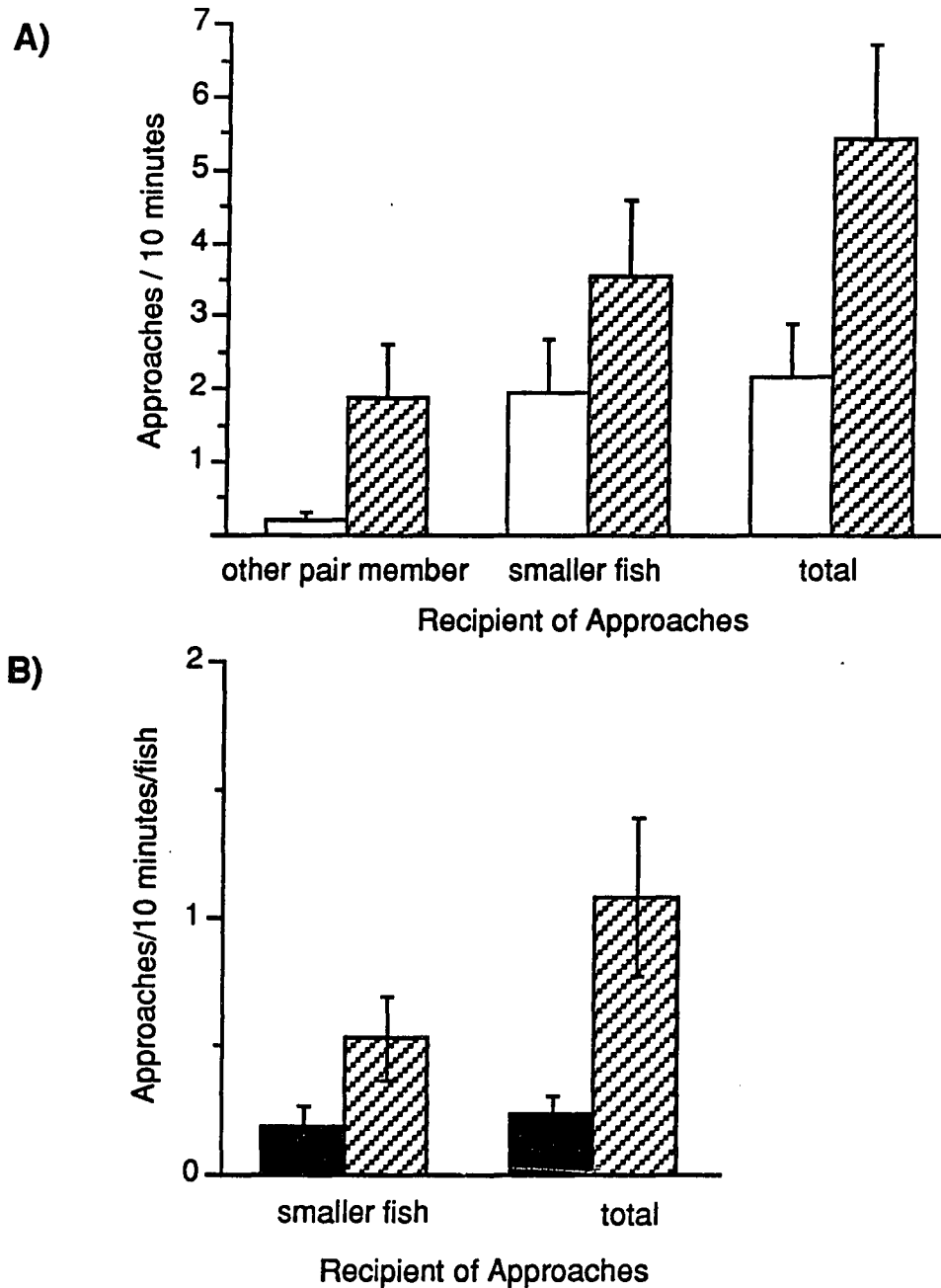
Figure 11. Approach behavior of unmanipulated males and females.
A) approaches per 10 minute sample on the other pair member,
smaller fish and total approaches; B) Approaches on smaller fish
and total approaches *per resident fish* per 10 minute sample (see text;
males shown in clear bars, females in cross-hatch).

Table 6. Summary of selected approach behavior by females, males, and advanced intersex fish in 1989 and 1991 samples (values are mean $\mp$ 1 S.E.M., sample sizes in parentheses).

Source	female → male	male → female	Total (by male)
unmanipulated pairs	1.85 $\mp$ 0.75 (20)	0.20 $\mp$ 0.09 (20)	5.40 $\mp$ 1.28 (20)
sex change baseline samples (1991)	2.14 $\mp$ 0.25 (32)	1.32 $\mp$ 0.22 (32)	5.88 $\mp$ 1.07 (32)
sex change baseline samples (1989)			9.68 $\mp$ 1.45 (20)
45 day samples (1989)			10.00 $\mp$ 3.20 (8)

significant (Mann-Whitney U test, 0.20 > p > 0.10). The comparison is still suggestive and difficult to account for as neither the number of other resident fish (unmanipulated pairs: 5.47 $\pm$ 0.64; sex change experiment pairs: 5.778 $\pm$ 0.572) or size difference between the male and female members of the pairs (unmanipulated pairs: 5.063 mm $\pm$ 0.927; sex change experiment pairs: 5.345 $\pm$ 0.677) was different between the two groups. Male approaches on females did not differ between the first and second baseline samples for the sex change experiments, none of the fish was handled prior to behavioral samples, and groups were located in the same general locations for both the unmanipulated pairs and sex change experiments, although these were not consistently randomized.

Behavioral changes with sex change.

1989 results. Frequencies of some of the behavior patterns described in Table 5 in the baseline male observations and over the course of sex change are shown in Figure 12. Behavioral changes were examined with the "time" and "behaviors X time" terms in repeated measures MANOVAs considering all recorded behaviors and periods from baseline (male) to 20, 30, and 45 days. No significant change occurred in the main effect of behavioral frequencies by 10 days into sex change, but did by 20 days and persisted in the 30 day comparion before decreasing at 45 days ("time" to 20 days: $F_{2,32}=6.68$, $p<0.01$; to 30 days: $F_{3,39}=4.39$, $p<0.01$; to 45 days: $F_{4,28}=2.20$, $0.10>p>0.05$). No significant changes in the relative proportions of each behavior from baseline were indicated when "bathe" was included in the analyses ("behavior X time" to 20 days: $F_{18,288}=2.18$, H-F adj. $p=0.095$; to 30 days: $F_{27,351}=1.89$, H-F adj. $p=0.0985$; to 45 days: $F_{36,252}=1.71$, H-F adj. $p=0.135$), but were at 20 and 30 days when "bathe" was not included (to 20 days: $F_{16,256}=3.97$, H-F adj. $p=0.002$; to 30 days: $F_{24,312}=3.30$, H-F adj. $p=0.003$; to 45 days: $F_{32,224}=2.02$, H-F adj. $p=0.057$). This result appeared to be due to the influence of the bathing data on covariance structure and consequent Huynh-Feldt degree of freedom adjustments. The significant interactions terms suggest differences between behaviors in pattern of change with time. Patterns with the main effect "time" did not change when "bathe" was dropped from analyses.

Approach was the most common behavior observed and the only one to show significant changes in frequency with sex change. Total approach frequency became significantly elevated over both male and ten day levels at 20 days (overall: $F_{2,32}=7.09$, $p<0.01$; contrasts: $F_{1,16}=17.57$, $p<0.001$ [male vs. 20d] and $F_{1,16}=6.58$, $p<0.05$ [10 vs. 20d]) and then showed a decreasing trend at 30 and 45
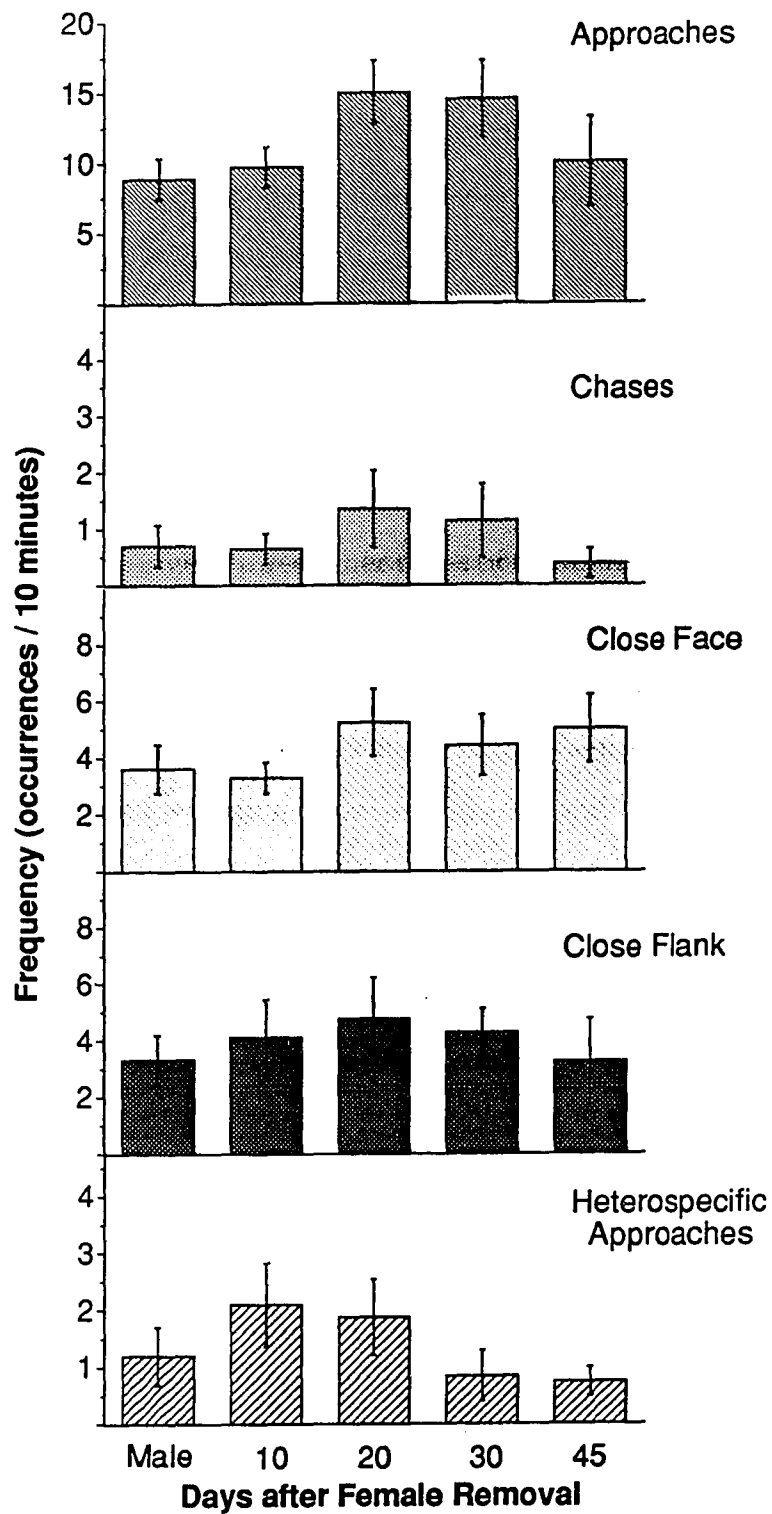
Figure 12. Behavioral measures from 1989 work in males (baselines) and various stages after female removal.

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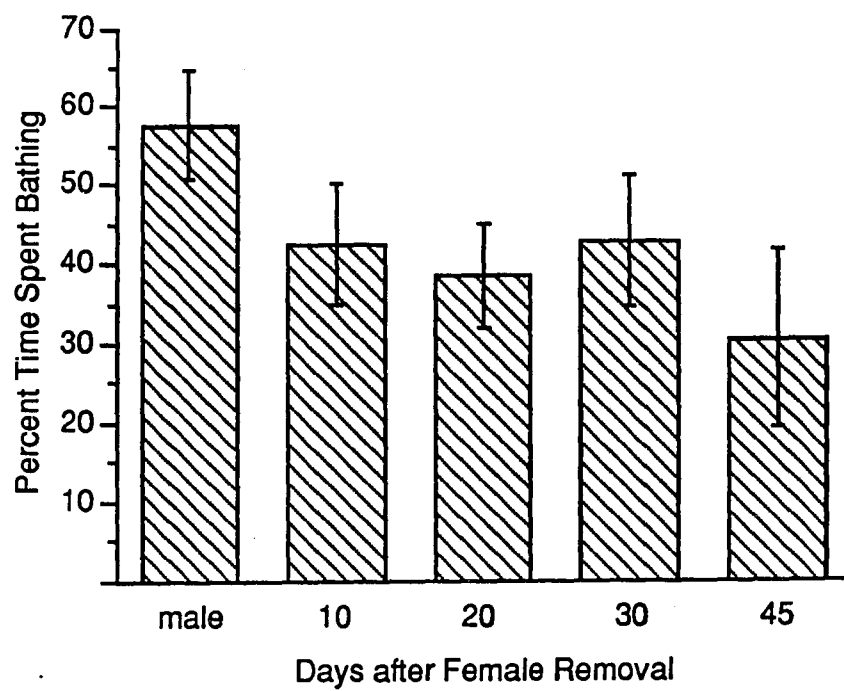
Figure 13. Proportion of time per 10 minute sample spent
bathing at different points after female removal.

days. Other agonistic behaviors such as "chase" and "close face" exhibited similar patterns to that of approach, but no statistically significant changes with sex change. No changes in frequency of bathing bouts was found, but the proportion of time spent bathing decreased significantly by ten days and remained lower than baseline levels for 45 days (Fig. 13).

1991 results

Behavioral sampling in 1991 focused on the shorter period after female removal from 1-20 days. Unlike the 1989 results, a highly significant increase over male levels was seen in total approaches by one day after female removal (overall comparison to 10 days: $F_{3,17}=4.60$, $p<0.01$, $n=20$; male vs. 1 day contrast: $p<0.005$; Figure 14). Total approach frequency declined after one day, but remained significantly elevated above male levels at 20 days ($p<0.05$, $n=12$). Aggression against the other member of the adult pair increased strikingly following female removal. Approaches by sex changers on their new male pair mates were significantly more frequent than approaches directed at females prior to their removal by one day. These levels then decreased in a similar manner to total approaches while remaining significantly elevated over male levels to 20 days (overall comparison to 10 days: $F_{3,57}=7.55$, $p<0.001$, $n=20$; to 20 days: $F_{4,48}= 6.45$, $p<0.001$, $n=12$; male < 1, 5 d: $p<0.001$; male < 10, 20 d: $p<0.01$). It was not always clear which individual would assume the male position prior to female removal, and insufficient data were obtained to compare the number of approaches received by these individuals pre- and post female removal. The mean for approaches on new male pair mates at one day appeared higher than either total approaches or approaches on smaller fish in baselines

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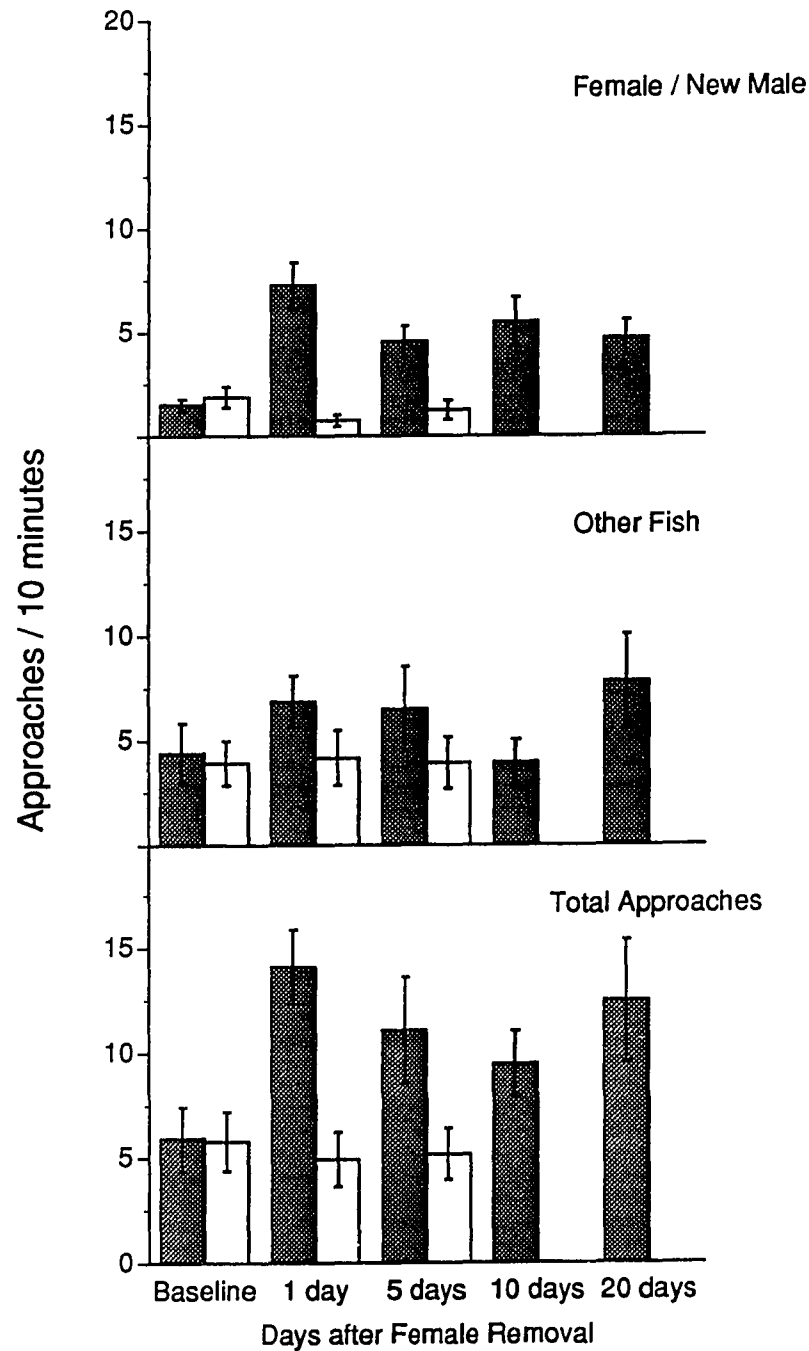
Figure 14. Approach frequency of males and at one, five, 10, and 20 days
into sex change (dark bars represent sex-changing fish, light
bars are handling control; n for 1-10 d = 18, 20d = 11).

(both conservative comparisons), but the differences were not significant. New males received a disproportionate number of sex changer approaches. Expected proportions for individual fish were generated by dividing total sex changer approaches by the number of other fish, including the new male, resident in a group. Fifteen of 17 new males which could be compared received more approaches from sex changers than expected (sign test of observed vs. expected proportions, p<0.005).

Approaches by sex changers on smaller fish other than the new male ("other fish") increased significantly by one day after female removal, then decreased as with the other approach measures (overall comparison to ten days in multivariate mode: $F_{3,17}=3.559$, p<0.05; male < 1d: p<0.05).

No differences in total approaches, approaches on females, or approaches on other fish were seen in the handling control experiment (n=12, $F_{2,22}=0.22$; $F_{2,22}=1.70$; $F_{2,22}=0.04$ respectively, P>0.05 for all three; Figure 14)

As with unmanipulated males, the total number of approaches exhibited for a given sex changer was positively correlated with the number of other fish resident in a sex changer's group ($r_s=0.611$, p<0.01, n=18). Total approach frequency was not correlated with body size in sex-changing fish.

Approaches received by males from females ceased with female removal at the beginning of manipulations. However, if approaches from the other pair member (females before their removal, new males after female removal) are considered, no change was produced by female removal ($F_{3,54}=0.463$, p>0.5). The lack of difference in this measure may be attributable to the high variability in female approaches on males mentioned earlier. Data on the sequence of approaches given and received were not taken. However, it subjectively

appeared that most approaches by the new males towards the dominant sex-changing individuals closely followed, and appeared to be precipitated by, approaches directed at them by the sex-changers. No change in the female approaches on males was seen following manipulation in the handling control experiment ($F_{2,22}=0.35$, p>0.5).

A striking increase in visit frequency was observed in most groups following female removal, although considerable variation was apparent (Fig. 15). This frequency showed a decrease from the one to 20 day samples similar to that for approaches. Since baseline measures were not obtained for most of the sex changers, three other types of comparisons were performed. i) No change in visit frequency occurred following manipulation in the handling controls ($F_{2,22}=0.99$, P>0.05). ii) Two-sample t-test comparisons of $\log_{10}(x+1)$ transformed data showed that baseline visit frequency in the handling control experiment was significantly lower than those from the sex-change experiments at one day (p<0.001) and five and ten days (p<0.05), but not 20 days (p>0.05). iii) Repeated-measures ANOVA showed a significant decrease in visit frequency in the sex-change experiment from one day levels to later samples (comparisons to ten days: $F_{2,30}=4.79$, p<0.01; to 20 days: $F_{3,30}=5.80$, p<0.01).
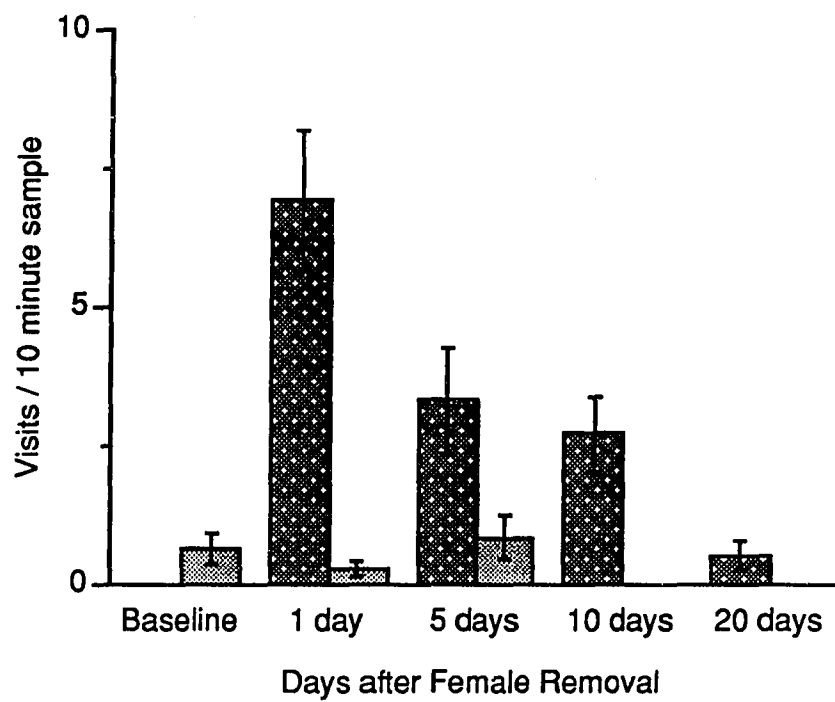
Figure 15. Visit frequency at various times after female removal
(dark bars: sex changers, light bars: handling control).

Simulated Territorial Intrusion Experiment

Figure 16 illustrates the results of the simulated territorial intrusion experiment. Seven individuals were followed to 45 days and underwent sex change in this experiment (five fish did not change sex: three had larger females take over their territories, one moved to a large female's territory, and one to the territory of a larger sex changing individual). The absolute number of attacks directed at the model intruder increased by 15 days while the numbers of attacks at the center and periphery of the territory became similar over a longer time course. Repeated measures ANOVA showed a significant increase in attacks with time (time effect: $F_{3,30}=5.80$, $p<0.01$), that attacks were greater at the center of the territory (distance effect: $F_{1,6}=11.87$, $p<0.05$), and that the relationship of attacks at the center to those at the periphery changed over the course of the experiment (time X distance interaction: $F_{3,18}=3.16$, $p<0.05$). Data were obtained for two of the males which did not change sex following takeover of their territories by larger females following removal of the original females. These males showed opposite changes in attacks on the model: one increased from 6 and 11 attacks (center, 1 m) to 46 and 39 by 30 days while the other decreased from 50 and 24 to 40 and 12 over the same period.

Interestingly, little or no aggression was directed at the model by fish who were resident in a given colony, but not members of the adult pair. These fish showed no apparent change in behavior, and were often feeding, while adult pairs were vigorously attacking the model intruder. This includes those fish who would become the new males (prior to female removal). The new males did show considerable aggression toward the model intruder in the first post-female removal samples (15 days), but data were not taken.
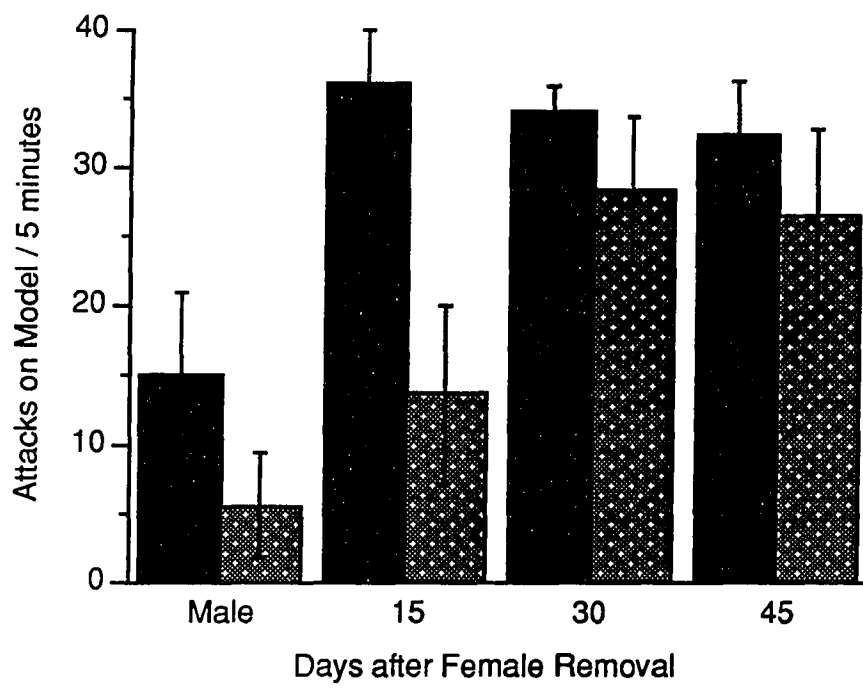
Figure 16. Attacks on model intruder at center and periphery of
territory (center: clear bars, periphery: stippled bars)

Correlations of steroid hormones with behavior

The hormonal correlates of aggressive behavior were examined in two ways: overall patterns and through correlations of steroid levels in individuals with frequency of approach behavior. As already described, females are more aggressive than males. Figure 17 shows the patterns of changes in plasma levels of Ad, T, 11-KT, E_2 and cortisol. Females had higher levels of Ad, T, and E_2 than males, but lower levels of 11-KT. Cortisol levels did not differ between the sexes.

In overall pattern, androgens and aggressive behavior changed in opposite directions after the initiation of sex change (Fig. 18). E_2 levels also correlated poorly with overall changes in approach frequency. E_2 showed no change until 20 days, when a significant increase in E_2 levels was seen. Cortisol levels did showed a qualitatively similar pattern of changes to approaches. However, while approach frequency peaked at one day following female removal and then declined, cortisol levels did not increase significantly until 20 days (20 days vs. all other groups: $p<0.01$).

Correlations of steroid levels and approach frequencies were more difficult to intrepret on an individual level. Table 7 shows Spearman correlations (r_s) among the measured steroids, number of other resident fishes, and three measures of approach frequency. Strong positive correlations between the number of other fish resident in a colony and approaches on fish other than the new male and, to a lesser extent, total approaches (new male included) were observed. A positive correlation existed between plasma cortisol and approaches on smaller fish. Trends suggesting positive relationships between i) number of resident fish and both plasma E_2 and cortisol and ii) plasma E_2 and approaches on smaller fish were also apparent. Neither 11-KT or Ad levels were correlated

with either number of fish or approaches. Unfortunately, too few measurements of both behavior and T were available for meaningful analyses.

The separate correlations between plasma levels of both E_2 and cortisol to the number of resident fish and to approaches on smaller fish were extremely similar (Table 7, Fig. 19). When the influence of number of resident fish on frequency of approach is controlled for by examining approaches/resident fish: the relationship between number of approaches on smaller fish and E_2 becomes weaker ($r_s=0.398$, $p>0.1$ for approaches on smaller fish), the trend between cortisol and total approaches disappears ($r_s=0.091$, $p>0.5$), and the correlation between cortisol and approaches on smaller fish becomes slightly stronger ($r_s=0.579$, $p<0.02$). This suggests there is no direct link between E_2 and aggression, while a cause or effect link with aggression appears likely for cortisol.
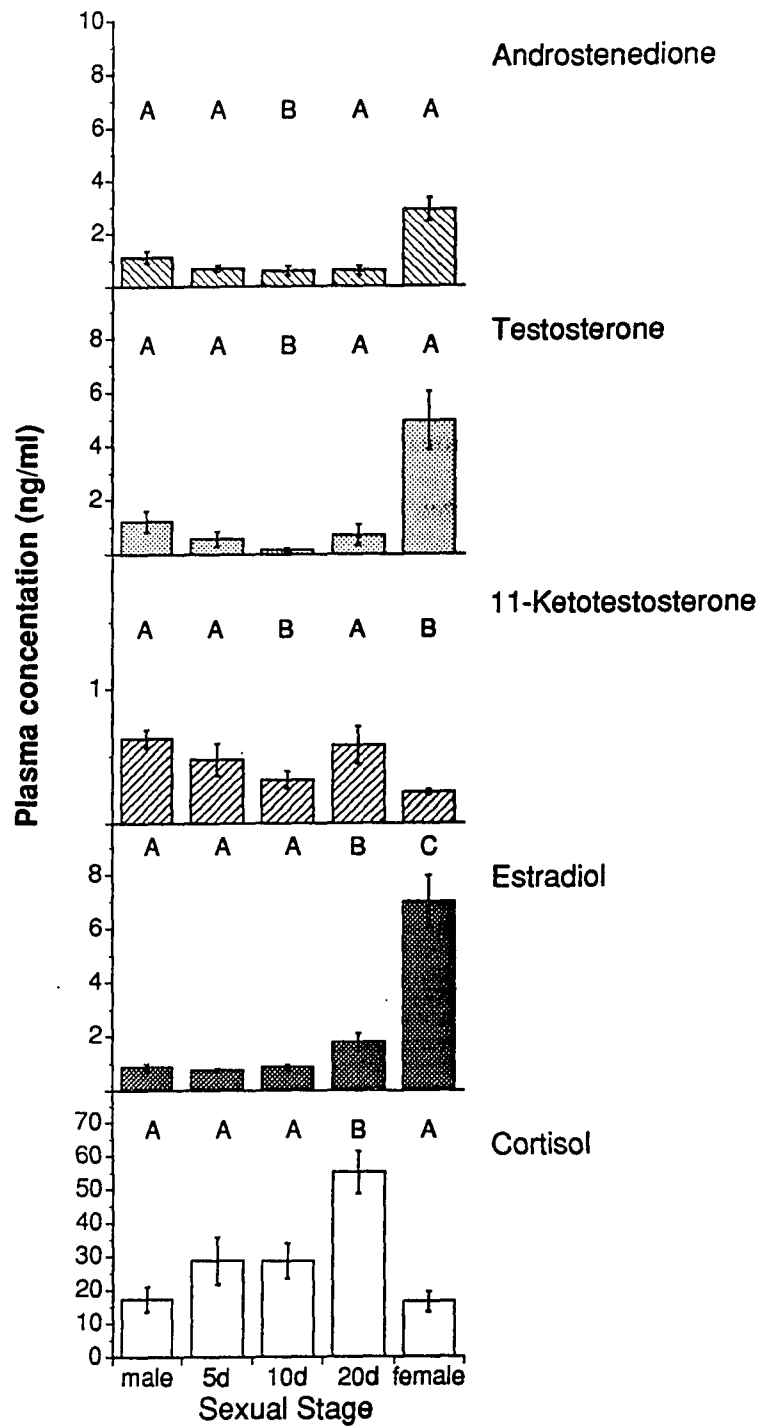
Figure 17. Plasma concentrations of steroid hormones in males, females, and intersexes of the anemonefish *Amphiprion melanopus* (means with the same letter are not significantly different)

Table 7. Spearman correlation matrix (r_s) for number of resident fish in colonies, approach frequencies, measured steroid hormones ([#]: 0.10>p>0.05, *: p<0.05, **:p<0.01, ***: p<0.001; sample sizes are shown in parentheses).

	Number of Resident Fish	Approaches on New Male	Other Approaches	Total Approaches
Total Approaches	0.556* (17)			
Approaches on New Male	0.271 (17)			
Other Approaches	0.740*** (17)	0.535* (18)		
Androstenedione	-0.293 (10)	-0.216 (11)	-0.491 (11)	-0.452 (11)
Testosterone	0.500 (3)	0.600 (4)	0.000 (4)	-0.211 (4)
11-ketotestosterone	0.223 (14)	0.082 (14)	0.220 (14)	0.073 (14)
Estradiol-17b	0.464[#] (17)	0.090 (18)	0.447[#] (18)	0.299 (18)
Cortisol	0.415[#] (17)	0.278 (18)	0.468* (18)	0.413[#] (18)
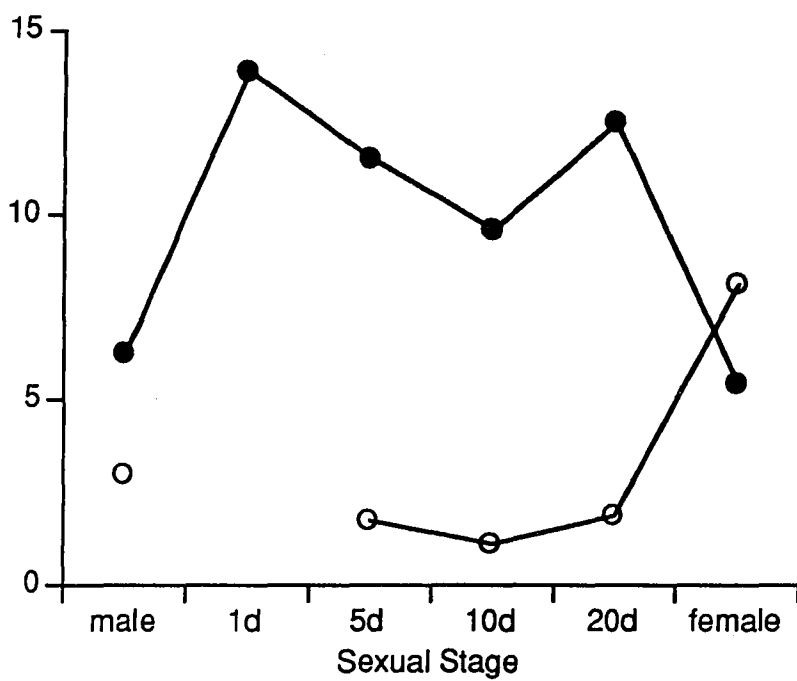
Figure 18. Mean total androgens vs. total approach frequency for males, females, and sex-changing fish (total androgens [ng/ml] shown in open circles, total approaches [#/10 mintes] are shown by closed circles).
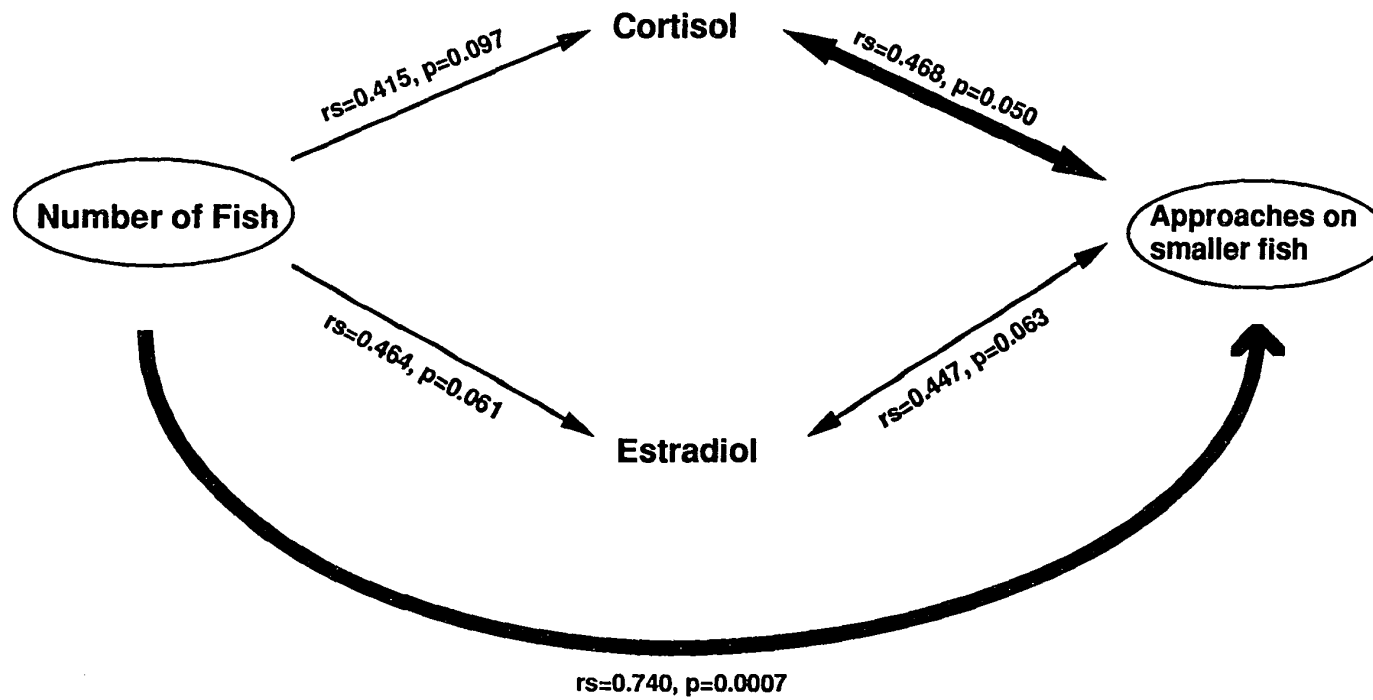
Figure 19. Correlation diagram for number of resident fish, approaches on smaller fish, and steroids (bold lines show significant correlations, thin lines show strong trends).

Discussion

Social Control of Sex Change

Social control of sex change in *A. melanopus* has been verified by the following observations: i) sex change was successfully induced or definitely initiated by female removal in a large number of manipulations in Papua New Guinea, Australia, and Guam (n=57), ii) sex change did not occur in males whose territories were taken over by larger females (n=7) or two males who moved to other territories where larger individuals were resident (a female and a sex-changer respectively; Godwin, in prep.).

While the exact nature of this social control is unclear, some potential cues have been identified. Males received about two approaches from dominant females per ten minute sample (see results), or roughly 150 per day, in observations of 52 pairs. This figure drops to zero following female disappearance and could be a critical cue for sex change. Fricke and Fricke (1977) suggested supression of sex change in males of *Amphiprion akallopisos* through aggressive dominance of females. The frequency of approaches from the other member of the adult pair, females prior to their removal and new male pair mates after female removal, did not change significantly with female removal in *A. melanopus* and is therefore probably not an important cue. The size or dominance status of the fish from which approaches are received and whether the recipient initiates aggression or not could be the important components of such interactions. Visiting behavior could also provide an important cue. This behavior has only been observed between members of mated adult pairs. The significant increase immediately after female removal and subsequent decline would be appropriate for initiating sex change. It is interesting that approaches

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by sex changers on new male pair mates often appeared to stimulate substrate-biting by these new males, a behavior usually associated with demersal nest preparation. Yanagisawa and Ochi (1986) reported that during pair formation after experimental *male* removals in another protandrous anemonefish, *Amphiprion clarkii* , substrate biting by the new males commonly followed attacks by resident females. They suggested this behavior was associated with the formation of dominance relationships. It should be noted here that Stahlschmidt-Allner and Reinboth (1988) found that olfactory cues may also be important in controlling sex change in *A. frenatus*, a close relative of *A. melanopus*. A complex suite of cues may control sex change on the reef.

Behavioral Changes in Sex Change

Apart from those directly associated with reproduction, sex-characteristic behaviors are not found in *A. melanopus*. The "signal jump" characteristic of male pomacentrids (Reese, 1964; Thresher, 1984) has not been observed in male *A. melanopus* or other *Amphiprion* species. This may be due to the sex-role reversal and monogamous mating pattern of anemonefishes. The lack of sex-characteristic behaviors precluded a qualitative assessment of behavioral sex change through the occurrence of secondary-sex behaviors possible with some protogynous species (e.g., "flutter runs" in *Labroides dimidiatus*, Robertson, 1972, 1974; "U-swims" in *Anthias squamipinnis*, Shapiro, 1981a). Behavioral sequencing was employed as a potentially sensitive method of detecting changes in behavioral profile in *A. melanopus*, but no consistent differences between pre- and post-female removal measures were found. This result may reflect the lack of control in the field over the number and size of resident conspecifics and interactions with other conspecific and heterospecific anemonefish.

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The rapid increase in aggressive behavior displayed by male *A. melanopus* following female removal appears to be a general characteristic of sex-change candidates following removal of secondary-sex individuals. The first behavioral samples in this study were not taken until a full day after female removal, but striking and significant increases in the number of approaches were evident. Increases in aggression may have occurred earlier. Robertson (1974) observed male-characteristic behaviors in as little as 53 minutes in *L. dimidiatus* . Warner and Swearer (1991) found that large females exhibited courtship and increases in aggression within several minutes of male removal in another protogynous wrasse, *Thalassoma bifasciatum*. Increases in aggressive and male-characteristic behaviors were not seen for 2-4 days in *A. squamipinnis* following male removal (Shapiro, 1981a). However, Shapiro's study may not be comparable with the two above studies on protogynous species because of differences in the nature and design of sampling or with field studies (Robertson, 1972; Warner and Swearer, 1991; and this study) since it was performed in aquaria.

Changes in other forms of aggressive behavior (e.g., close face, chases) paralleled those of approaches in the 1989 results (Fig. 12). The significant increase in approaches over male levels at twenty days, but not ten days, is difficult to account for in light of the 1991 results. It appeared to relate to an oceanic swell which increased water motion that seven individuals were exposed to during their ten day samples. My subjective impression is that these conditions decreased overall activity of the fish. The decrease in time spent bathing after female removal indicated an overall increase in activity after female removal since the fish are either feeding above the anemone or interacting with other group members when not bathing.

Given the variability found between individual *A. melanopus* , it cannot be stated at what point following female removal a sex-changer attains a "female-characteristic" behavioral profile based on aggressive behavior. Both the variation between individuals and the positive correlation of aggressive behavior with the number of other resident fish in a group point to a strong dependence of individual behavior on social environment. The simulated territorial intruder experiment did indicate significant changes in aggression directed towards a conspecific model intruder both in the overall increase of aggression within 15 days and in the relationship of aggression at the periphery of the territory to that at the center within 45 days. This result may indicate a change in the aggressive behavior of individuals from a male to female pattern with sex change. The MANOVA results indicating a change in overall behavioral profile by 20 days in 1989 support this interpretation. However, since the territorial intrusion experiment was not controlled, the occurrence of learning or some form of habituation affecting response to the model cannot be excluded. Arguing against this explanation for the present results, Ross (1976) found the opposite pattern in presentations of a model conspecific intruder to two adult *A. melanopus* pairs in Guam. Attacks on the model *decreased* 44 and 76% over periods of 13 and 45 days respectively. The lack of response of all subadult members of groups to the model intruder, and display of aggression towards the model by the new male (formerly α-subadult) following female removal, was intriguing. It suggests that territorial defense may present no net benefit if an individual is not reproductively active in a group.

Increases in aggression following the disappearance of secondary sex individuals may serve to prevent other initial-sex individuals in a group or area

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from changing sex and becoming competitors for mates rather than potential mates themselves. The group member other than the male most likely to change sex following female removal in *A. melanopus* is the α-subadult (which becomes the new male). One day after female removal, these individuals received significantly more sex changer approaches than expected by chance. The threat of territory takeover following removal or disappearance of secondary-sex individuals also appears to be important in *A. melanopus* and at least two protogynous species. Robertson (1974) found that territorial incursions by neighboring males began almost immediately following male removal in *L. dimidiatus*. In *Dascyllus aruanus*, males who were larger than all resident females and smaller than removed males took over territories in 11 of 22 manipulations performed by Coates (1982). Under certain conditions, a similar phenomenon also occurs in *A. melanopus* (Godwin, in prep.).

Hormonal Correlates of Behavior

Female *A. melanopus* are more aggressive than males. Potential hormonal correlates of this behavioral difference were identified in this work, but may be more simply explained by the needs of gonadal function. 11KT and T are important steroids for male reproductive function in teleosts (Fostier *et al.*, 1983). Hypothesized roles for 11KT in control of male reproductive and aggressive behaviors have received support from studies showing differences in circulating and gonadal production of T and 11KT between alternative male phenotypes in two protogynous species. Higher 11KT levels were observed in the more aggressive, territorial morphs (Cardwell and Liley, 1991b; Hourigan *et al.*, 1991). The only information on plasma steroid levels in a pomacentrid fish is from male *Chromis dispilus*, where courtship and spawning are associated with higher T

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levels (Pankhurst, 1990). From a behavioral standpoint, female *A. melanopus* would therefore be predicted to have higher androgen levels than males given their social dominance and more frequent aggressive behavior. Additionally, increases in plasma androgens during sex change would be expected based on the significant increase in aggressive behavior. These predictions were not well supported. Males have significantly higher levels of circulating 11KT. Females do have higher levels of Ad and T, but T and Ad levels are tightly and positively correlated with E_2 levels and both are biosynthetic intermediates in E_2 production. Moreover, a number of female teleosts including one protogynous wrasse exhibit higher T levels than males (e.g., Nakamura *et al.*, 1989; Prat *et al.*, 1990). Neither Ad or 11KT was correlated with observed approaches or number of other fish resident in a colony on an individual level. Most importantly, all three measured androgens *decreased* initially during sex change; a pattern opposite that shown by aggressive behavior (Fig. 18).

The results were less clear for E_2 and cortisol. E_2 levels were expected to increase with assumption of female function and would therefore provide an appropriate cue for increases in aggression at the initiation of sex change. This hypothesis was not supported. No increases in circulating E_2 were seen until 20 days after female removal. The trends towards positive correlations of E_2 and both number of other fish and approaches suggested a link of E_2 to behavior and social interactions. However, the strong correlation between number of fish and approaches, and the decrease in the strength of the relation between E2 and approaches when number of approaches was corrected for "opportunity" (number of fish), argue this is an indirect link. Females do have much higher E2

levels than males and influences of this steroid on mature female behavior may occur.

Cortisol is primarily a stress hormone in teleosts, but is also important for energy mobilization and other functions (Mazeaud et al., 1977). Males would be predicted to have higher cortisol levels than females in relation to their subordinate status as described for swordtails (Hannes, 1985). This was not the case. Cortisol levels showed an increase during sex change, perhaps reflecting an influence of heightened aggressive interactions. The suggestive relationship of cortisol levels with the number of other resident fish and positive correlation with approaches on smaller fish at the individual level was also supportive of a link between this steroid and social interactions. While the cortisol peak did occur well after that of approaches and the early elevation over male levels was not significant, early influences from social interactions and later influences related to energy mobilization for gonadal transformation (as suggested in Chapter III and Godwin and Thomas [in prep.]) could both be important.

The occurrence of socially-controlled sex change demonstrates a profound interaction between social interactions and reproductive physiology. Interpretation of cause and effect regarding sex steroids and behavioral changes is therefore difficult. This point is illustrated by the "web" of correlations between number of resident fish, approach frequency, and E_2 and cortisol levels (Fig. 19). Analysis of these correlations was not pursued further here precisely because the variables could not be confidently assigned as either predictor or response.

An underlying assumption common in behavioral endocrinology is that behavior represents an effect rather than cause of hormonal changes. An alternate hypothesis for the A. melanopus system could postulate that increases in

approach frequency are produced by processes related to social reorganization and/or change in the dominance status of the sex changer, with alterations of circulating steroids and perhaps even sex change being physiological results rather than causes. It is pertinent in this context that both gonadal and statistically significant endocrine changes lagged behind behavioral changes during sex change in *A. melanopus*. Warner and Swearer (1991) cited the rapidity of behavioral change (minutes) in *T. bifasciatum* as evidence against a causal role for hormones in the immediate behavioral shifts they observed. Since male anemonefish do not become dominants without changing sex, separation of these alternatives would require experimental prevention of sex change following female removal or its stimulation in the presence of a dominant female.

The sex-role and steroid patterns described here are similar to those of polyandrous birds. As with *A. melanopus*, a sex-steroid basis for behavioral role reversal has not been established in these species (e.g., Rissman and Wingfield, 1984; Gratto-Trevor *et al.*, 1990), although potential sex differences in brain aromatization and receptor levels remain largely uncharacterized (but see Schlinger *et al.* 1989). Potential reversals of other hormones, such as that found for prolactin in polyandrous spotted sandpipers (Oring *et al.*, 1986), should be investigated. Androgen increases associated with gonadal change could provide appropriate cues for aspects of male aggressive and reproductive behavior during sex change in protogynous fishes. In contrast, social dominance and male reproductive function are uncoupled in sex-role reversed species. During protandrous sex change, androgen increases could interfere with ovarian development, making these steroids poor cues for aggressive behavior in species such as *A. melanopus*. The results of this study are consistent with the view that

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the utilization of steroid hormones as behavioral cues is evolutionarily flexible and that the requirements of gonadal function may limit alterations of steroid secretion possible for behavioral control (Crews and Moore, 1986; Kelley, 1988; Moore, 1991).

CHAPTER V

DISCUSSION AND CONCLUSIONS

In contrast to studies of the ultimate causation and evolution of sex change, studies of proximate physiological causation of sex change to date have been hampered by the lack of a theoretical framework. Creation of such a framework has not been possible until recently as baseline information upon which to formulate hypotheses was not available. Providing such information for a promising model system was one objective of this project. In addition, recent studies of sex change in the protogynous wrasses *Thalassoma duperrey* (Ross *et al.*, 1983; Ross, 1984; Nakamura *et al.*, 1989; Hourigan *et al.*, 1991) and *T. bifasciatum* (Warner and Swearer, 1991; Koulish and Kramer, 1989; Grober and Bass, 1991a,b) and the elegant work of Cardwell and Liley (1991a,b) on the protogynous stoplight parrotfish *Sparisoma viride* make these attractive systems for examining the proximate control of sex change.

A framework for the study of sex change in teleosts based on the organization-activation theory of hormone action (Phoenix *et al.*, 1959; Young *et al.*, 1964) is proposed here. A link between this construct and general vertebrate sex determination and sexual phenotype development is suggested. This is followed by a critique of some recently-published sex change experiments and suggestions for the design of future experiments to improve interpretation and relevance to sex change as it occurs naturally. Finally, experiments designed to test hypotheses relating the proximate causation of sex change in *A. melanopus* to hormone action and examining the relationship between behavioral and physiological changes are proposed. If sex change can be blocked when it should

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occur or stimulated when it should not occur, the links between behavioral and physiological components of sex change can be investigated.

Organization-Activation Theory and the Physiological Basis of Sex Change.

The now classical separation of the actions of steroid hormones into organizational and activational influences (Phoenix *et al.*, *1959*; Young *et al.*, 1964), or permanent and impermanent (Arnold and Breedlove, 1985), provides a useful framework with which to examine teleost sexuality. This conceptual framework is still applicable if steroid hormones prove to be either not involved or not the sole or direct influence(s) affecting sexual development. Francis (1992) has argued that sexuality in teleosts is best viewed as a continuum with strict gonochores at one end, simultaneous hermaphrodites at the other, and species showing sexual plasticity as juveniles (sex determination) and adults (functional sex change) in between. This continuum represents a decreasing canalization of the development of germ cells into either male or female function. Cardwell and Liley (1991a) addressed this issue for the protogynous *S. viride* and suggested that either the period of sensitivity to steroid hormones has moved to a later point in life (an organizational or permanent influence) or that sexuality is sensitive to hormonal environment throughout life (an activational or impermanent influence). Two male morphs are found in *S. viride*. One is brigtly colored and territorial, the other is drably colored and non-territorial. Because sex and color change are not tightly coupled in this species, steroid influences on each process could be dissected. The observation that 11-ketotestosterone (11-KT) levels rose with the initiation of sex change in *S. viride*, then fell when male function was assumed but no change of color had taken place, suggests an organizational rather than activational influence of this steroid on gonadal sex.

The direction of sexual development was irreversibly changed, but elevated levels of the causative agent were not necessary to maintain this change. Evidence of activational influences of this steroid on behavior in this species was provided by observed differences in 11-KT levels in males of different social status, and changes in these levels following a territorial challenge (Cardwell and Liley, 1991b).

Ideally, a model of sex change in teleosts should be based on general patterns of teleost and vertebrate sexual development. This requires that functional sex change be viewed as a second sexual maturation occurring in the lifetime of an individual. Is this interpretation justified?

Cytologically, the initiation of sex change is characterized by a cessation of initial sex function. The first histologically-discernible indicator of protogynous change in most species is oocyte atresia (Sadovy and Shapiro, 1987). The exceptions are the unusual cases seen in some protogynous gobies which pass through a simultaneously-hermaphroditic stage (Cole, 1990). The typical degeneration of ovarian tissue at the onset of sex change suggests a cessation of critical support for vitellogenic processes (Cochran and Grier, 1991). Two possibilities for this support are gonadal steroids and gonadotropins. Estradiol-17β (E$_2$) is known to be important for vitellogenesis in a number of teleosts (Ng and Idler, 1983) and is also known to decline at the initiation of sex change in several species (Nakamura et al., 1989; Cardwell and Liley, 1991a; Cochran and Grier, 1991; but see Yeung and Chan, 1987a,b). As noted in chapter III, A. melanopus shows a pattern of change in gonadal steroids during sex change which is functionally similar to that described for several protogynous species, but reversed temporally since this species is protandric.

The sex of an individual in a period of gonadal regression may be in a plastic state and susceptible to sex inducers. Gonadal recrudescence might then proceed in either the male or female direction depending on these sex inducers. Fishelson (1975, p. 293) suggested a similar pattern and seasonal component for protogynous change in *Anthias squamipinnis*, but invoked an antagonistic "block" on spermatogonial proliferation by ovarian activity and estrogens. Development of spermatogenic tissue coincident with a cessation of ovarian activity was observed in females of the gonochoristic surgeonfish *Acanthurus nigrofuscus* at the end of the breeding season (Fishelson *et al.*, 1987). The spermatogenic tissue disappeared with the onset of ovarian maturation in the next breeding season and no evidence of functional sex change was observed. That sex change is not a necessary consequence of gonadal regression is shown by patterns in some temperate sex-changing species. The protogynous temperate black sea bass, *Centropristes striatus*, undergoes gonadal regression annually, but only about 14% of females in the population change sex in a given year (Wenner *et al.*, 1986 cf. Cochran and Grier, 1991).

If the view of adult sex change as a second sexual maturation is accepted as a working hypothesis, then an organizational action of putative inducer(s) on the gonad during secondary-sex development which is consistent with our knowledge of both sex change and teleost sexual development generally can be proposed. Sex is extremely manipulable in gonochoristic fishes by the administration of exogenous sex steroids, but only prior to maturation (reviewed in Hunter and Donaldson, 1983). Sex changers may be similar in that sexual plasticity is maintained prior to the initiation of secondary sex function, but is lost following sex change. In contrast to many invertebrates, no species of fish is

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Table 8. Experiments on precocious sex change induction in protogynous species through administration of sex steroids and other hormones.

Species	Common name	hormones administered	Sex Change?	Authors
Thalassoma bifasciatum	Bluehead wrasse	T	yes	Stoll, 1955
Coris julis	wrasse	T	yes	Reinboth, 1962
Halichoeres poecilopterus	wrasse	androgen	yes	Okada, 1962
Sparisoma viride	Stoplight Parrotfish	11KT	yes	Cardwell and Liley, 1991a
Anthias squamipinnis	Fairy Basslet	T	yes	Fishelson, 1975
Monopterus albus	Ricefield Eel	T, MT, 11KT	no	Tang et al., 1974
		mammal LH	yes	"
Thalassoma bifasciatum	Bluehead wrasse	HcG	yes	Koulish and Kramer, 1989

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known to be capable of reverting to the initial sex following sex change. The goby *Paragobiodon echinocephalus* may be an exception, but this conclusion awaits more information (Kuwamura, 1991). Sex change then is one-way. As with juveniles of gonochoristic species, sex change can usually be precociously induced in hermaphroditic species by exogenous sex steroids (Table 8; Cardwell and Liley, 1991a and references therein). The only attempt at causing a reversion to initial sex, by administration of E_2 to males of the protogynous *Monopterus albus*, resulted only in destruction of testicular lobules (Tang *et al.*, 1974a). More manipulative studies of this type would be valuable to better establish the irreversibility of sex change.

Other similarities between juvenile gonochores and initial-sex individuals in sex changers include the presence of gametogenic tissues of both sexes in the gonad in many species (gonochores: Takahashi, 1977; Takahashi and Shimizu, 1983; Tricas and Horimoto, 1989; Buxton and Garratt, 1990; Naish and Ribbink, 1990; sex changers: Sphigel and Fishelson, 1986; Chan and Phillips, 1967; Yeung and Chan, 1987). No trace of functional initial-sex tissue is apparent following sex change. Simultaneous hermaphrodites and species which pass through a simultaneously hermaphroditic stage do not fit neatly into this scheme. In such cases, it may be necessary to either invoke more localized hormonal actions than are commonly conceived of or mediation of sexual development by some other process.

The model proposed to this point postulates that secondary-sex function in sex-changing species is induced through organizational actions which permanently direct development in the direction of the secondary sex. The primary difference between gonochoristic species and those which undergo adult

sex change may simply be the point in ontogeny at which this event takes place and sexual development becomes canalized: prior to or following sexual maturation respectively. The nature of sex inducers and developmental mechanisms may be identical. Except for the proposed organizational influence, this idea has already been suggested by Francis (1992).

What about initial-sex development in sex-changing species? A retention of the sexual plasticity characteristic of immature gonochores suggests that influences controlling initial-sex function act activationally. The fact that change to secondary-sex function occurs rules out a permanent action for the influences responsible for initial-sex function. The model is further developed below to propose the induction of initial-sex development through activational influences.

It appears that initial-sex function is facultative in at least some sex-changing fishes. The best evidence is from *Amphiprion* species. Godwin (Chapter II, in prep.) found that the gonads of juvenile, non-breeding *A. melanopus* consist primarily of immature ovarian tissue, rather than the mixture of testicular and ovarian tissue seen in functional males. Other workers have found that extensive testicular development occurs in *Amphiprion* species only if an individual assumes the male breeding position (*A. frenatus*; Stahlschmidt-Allner *et al.*, 1988; *A. clarkii*: Hattori and Yanagisawa, 1991). In a related protogynous pomacentrid, *Dascyllus aruanus*, direct maturation as a male appears to occur if an individual is the largest member of its social group (G. E. Forrester, pers. comm.). One population of this species sampled in Guam exhibited a 1:1 sex ratio, suggesting direct maturation into male function for some individuals (Godwin, unpubl. data). Diandric patterns are well known from labrids and scarids (wrasses and parrotfish). The best information on population-level patterns of diandry comes

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from studies of the wrasse genus *Thalassoma*. In *T. bifasciatum*, the proportion of individuals which are primary males (differentiated directly as males without an intervening female phase) on reefs is positively correlated with population density (Warner, 1980), perhaps implying that female development is facultative in this group also (see Shapiro, 1992).

Facultative development of primary-sex gonadal tissue indicates a flexible rather than strongly-canalized developmental system. This development could be controlled by activational influences of primary sex inducers. Shapiro (1992) hypothesized that male sexual development in protandrous species was produced by a temporary "masculinizing influence" superimposed on an underlying female path of development (the word "activational" could easily substitute for "temporary" here). The demonstrated decrease in all androgens at the initiation of sex change and persistent decrease for 11-KT in *A. melanopus* with change to female function shown in chapter III support both Shapiro's hypothesis and the more general hypothesis proposed here. The necessary tests would involve experimental manipulation of the assumption and maintenance of initial-sex function. In *A. melanopus*, data on changes in androgen levels with the assumption of male function from the immature state, experimental prevention of this transition, and disruption of male function would all address this question.

Sex Change Experiment Critique and Suggested Designs

Reproductive physiology is intimately linked to behavioral interactions and other social cues in species with socially-controlled sex change. It follows that social influences should be considered when manipulative endocrinological studies of sex change are performed in these species. Sex change has been

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successfully induced in several protogynous species by administration of exogenous hormones including human chorionic gonadotropin (hCG), ovine luteinizing hormone, and gonadal steroids (Table 8). Social control of sex change has been demonstrated in two of these species, *A. squamipinnis* (Fishelson, 1970) and *T. bifasciatum* (Warner and Swearer, 1991), and is likely in *S. viride*, *Halichoeres poecilopterus*, and *Coris julis*. The successful inductions of sex change in several of these experiments provide valuable information about potential influences on sex change. They present difficulties of interpretation, however, because experimental fish were either isolated, eliminating social interaction, or in social situations in which one or more sex changes might be expected without hormonal treatment. These difficulties are illustrated by examples from two of the studies in Table 8.

Isolated female *T. bifasciatum* injected with human chorionic gonadotropin exhibit sex change significantly more frequently than control females (Koulish and Kramer, 1989), suggesting native gonadotropin(s) may be involved in the stimulation of sex change. Putting aside questions of heterologous preparations, can it be concluded that such a mechanism is important in nature? In a congener of *T. bifasciatum*, *T. duperrey*, the proximate control of sex change under experimental conditions is critically dependent on stimulation from smaller fish and inhibition from larger fish (Ross *et al.*, 1983). Warner and Swearer (1991) found that sex change by female *T. bifasciatum* in the field was stimulated by removal of large males. The question left open by Koulish and Kramer's study then is: would females injected with hCG change sex in the presence of a large male? Elevated gonadotropin(s) could be a necessary, but not sufficient, condition for the initiation of sex change in the natural situation.

The second design involves placing a number of females together: some are administered a hormone, others are not and serve as controls (Cardwell and Liley, 1991a). A variation in which all females are administered the hormone in aquarium water has also been used (Fishelson, 1975). The difficulty arises from the expectation of sex change in such groups without hormonal manipulation. In groups of unmanipulated female *A. squamipinnis*, sex change is observed in the largest female following male removal (Fishelson, 1970; Shapiro, 1981a). Multiple, simultaneous sex changes are observed in groups of female *T. duperrey* (Ross *et al.*, 1990). A more appropriate control for an experiment like that of Cardwell and Liley (1991a) would therefore be a second group of females who were sham-injected.

A second issue regarding the interpretation of manipulative sex change experiments is that of cause and effect. Coincident changes in sex steroids and gonadal histology may indicate that the steroids are secondary effects rather than causal influences. Similarly, exogenous steroids may stimulate sex change in some species through alterations of some other physiological factor rather than by direct effects on gonadal cells. Taking the experiment of Cardwell and Liley with *S. viride* as an example, 11KT could act directly on gonial cells to initiate spermatogenesis or indirectly through feedback effects on the pituitary or hypothalamus. Grober and Bass (1991a,b) found that terminal-phase male *T. bifasciatum* possessed higher numbers of Gonadotropin-releasing-hormone (GnRH) cells than females or initial phase males, and that an increase in numbers of these cells could be stimulated through injections of 11KT. Perhaps 11KT stimulates sex change in *S. viride* through increases in numbers of GnRH cells and resultant increases in circulating gonadotropin(s).

A convincing demonstration that a given factor plays a critical role in the stimulation of gonadal change would require both correlative evidence of its importance in natural sex change and induction of the process under social conditions where it should not occur. If the presence of larger individuals is supressing levels of a critical physiological factor (e.g., 11KT or gonadotropin), circumventing this supression by artificially increasing levels should cause sex change in the presence of a large individual. An unfavorable social environment may present an endogenous "block" at some point in the physiological pathway leading to sex change. If social circumstances are not taken into account, it cannot be known whether a factor (e.g., 11KT) exercises its influence prior to or following this block (Fig. 20). Experimentally, addressing this would involve stimulating sex change in the presence of a male for most protogynous species. Conversely, preventing sex change following male removal through the use of specific inhibitors of a given factor would also provide strong evidence of its influence. The key element is the critical role of social interactions in the process.

Sex Change - Experimental Designs

A number of potentially-important steroidal influences on protandrous sex change in *A. melanopus* were identified in chapter III. Experimental examinations of steroid and gonadotropin influences designed within the organization-activation framework and with the concerns of the previous section in mind are outlined below and briefly summarized in Table 9.
These are intended as examples, rather than detailed descriptions for experiments. The same experiments could easily be modified to examine protogynous change. The hypotheses below may be too simplistic, but complex models cannot be justified before simpler ones are rejected.
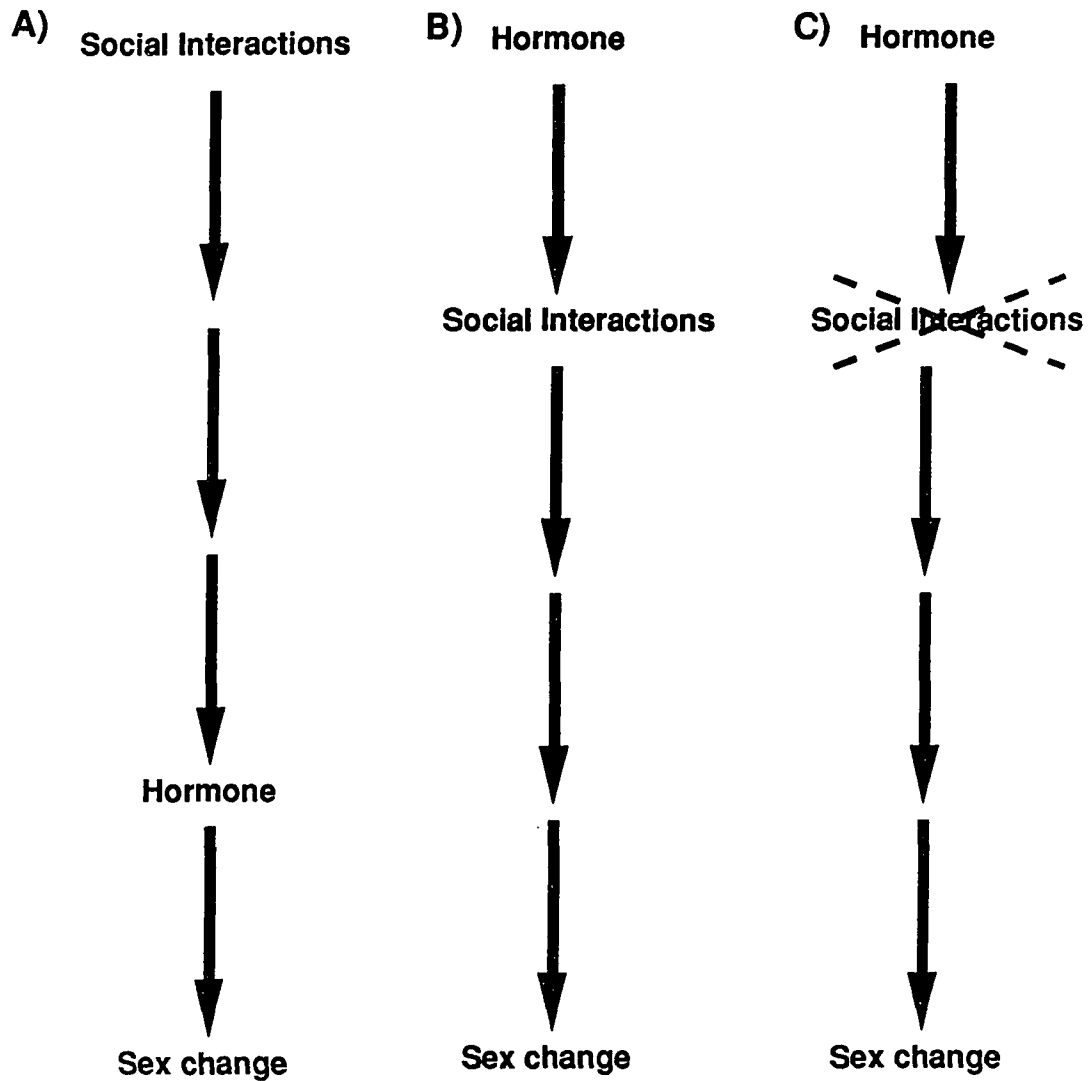
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Figure 20. Possible pathways in the control of sex change and pitfalls for
experimentation. A) response to steroids unaffected by social
interactions, B) response to steroids mediated by social
interactions, C) social interactions removed, misinterpretation
of steroid effects results.

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Hypothesis #1: Androgens act activationally to stimulate the development of spermatogenic tissue in *Amphiprion*. The decline in these hormones at the beginning of sex change causes the cessation of spermatogenic tissue development and permits sex change to proceed. Rising androgen levels are responsible for spermatogenic development when an individual assumes the male breeding position.

Predictions: Artificially increasing androgen concentrations should prevent sex change by males after female removal. Decreasing androgen action either with a general steroidogenesis inhibitor or through androgen receptor blockers should induce the decline of spermatogenic tissue and sex change in the presence of a female. Administration of androgens to subadults should produce male-characteristic gonadal histology while blocking androgen action should prevent the development of male function under conditions where it should occur (ie., when a subadult assumes the male breeding position).

Hypothesis #2: Rising E_2 levels exert an organizational influence during sex change, stimulating oogenic tissue development and gonadal change. [This hypothesis was discounted in chapter III based on the time course of gonad development and E_2 increases in sex change, but caveats were noted.]

Predictions: Implanted E_2 will stimulate sex change without female removal, while an E_2 receptor blocker will prevent it after female removal. Sexual plasticity will be lost following sex change.

Hypothesis #3: An increase in the E_2/androgen ratio exerts an organizational influence and changes the course of development from spermatogenesis to oogenesis, stimulating sex change.

Predictions: Artificially increasing the E_2/androgen ratio through implantation of differing amounts of E_2 and androgens while blocking endogenous steroidogenesis will induce sex change. Blocking an increase in the ratio with aromatase inhibitors (which block the conversion of testosterone to E_2) will prevent sex change following female removal.

Hypothesis #4: Increases in cortisol after female removal stimulate gonadal change and therefore sex change.

Predictions: Implanted cortisol will induce sex change without female removal. A cortisol synthesis blocker such as RU48386 will prevent sex change following female removal.

Hypothesis #5: Sex change is stimulated through an increase in plasma gonadotropin levels (see Koulish and Kramer, 1989; Tang *et al.*, 1974b), which are indirectly stimulated by increases in GnRH output (see Grober and Bass, 1991a,b).

Predictions: Artificially increasing levels of either GnRH or gonadotropin(s) will induce sex change. A gonadotropin-synthesis inhibitor such as methallibure will prevent sex change following female removal.

A critical part of these experiments would be plasma steroid measurements after manipulation to insure that treatments had the desired effect. Steroid measurements from gonadal tissue might also be informative.

Table 9. Experimental tests of the role of steroid hormones in protandrous sex change in *Amphiprion melanopus*.

Hypothesis	Treatments	Female Removal?	Sex Change? (Prediction)
A. Decreasing androgens produce decline in testicular tissue at beginning of sex change, allowing ovarian tissue to develop.	1. implant androgens	yes	no
	2. implant cyproterone acetate (steroidogenesis inhibitor)	no	yes
	3. implant androgen receptor blockers	no	yes
B. Increasing estrogens stimulate sex change through increases in ovarian tissue	1. implant E_2	no	yes
	2. implant E_2 receptor blocker	yes	no
C. Increasing estrogen/androgen ratio after female removal initiates decline of testicular tissue and development of ovarian tissue	1. implant aromatase inhibitor	yes	no
	2. implant cyproterone acetate and E_2 to increase ratio	no	yes
	3. implant cyproterone acetate and both E_2 and androgens to maintain ratio at male levels	no & yes	no no
D. Increases in cortisol during sex change stimulate gonadal change.	1. implant cortisol	no	yes
	2. implant cortisol antagonist	yes	no
E. Increasing GnRH/gonadotropins stimulate gonadal change	1. implant gonadotropins, GnRH	no	yes
	2. implant gonadotropin blocker	yes	no

A)

Female Removal

CNS

perception

"Aggression"
Center

hypothalamus

Aggression effectors

Pituitary

Gonadotropins(?)

Gonadal steroids

Gonads

×

Sex Change

B)

Female Removal

CNS

perception

"Aggression"
Center

hypothalamus

Aggression effectors

Pituitary

Social interactions

Gonadotropins(?)
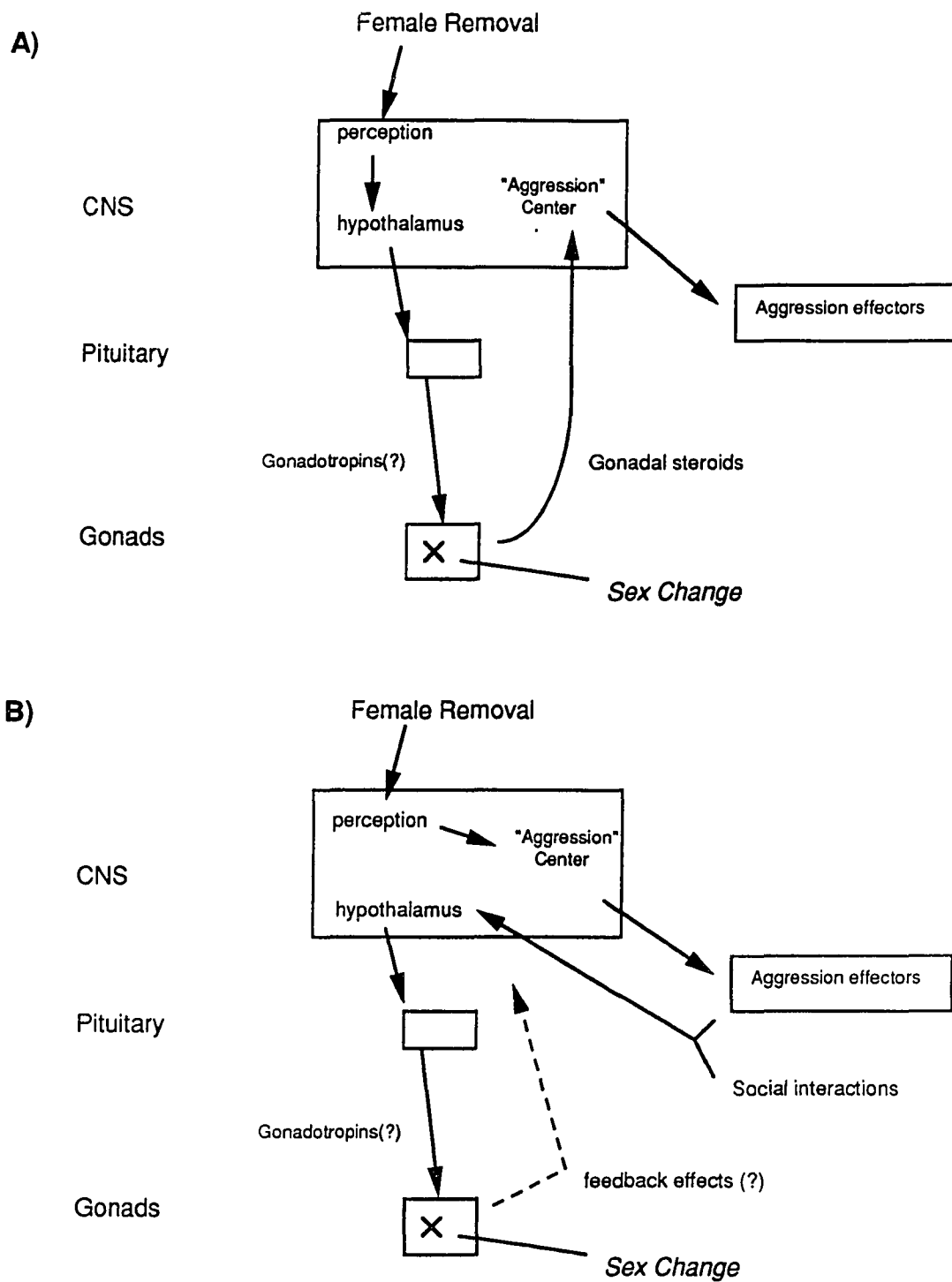
feedback effects (?)

Gonads

×

Sex Change

Figure 21. Schematic of models with (a) behavioral changes as a result of sex change, and (b) behavioral changes driving sex change.

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Relationship between behavioral and physiological changes

A direct benefit of experiments like those above would be realized if gonadal change could either be prevented following removal of a dominant individual or induced without removal. This would permit separation of the behavioral and physiological components of sex change and experimentally address the question posed in chapter IV, "Are behavioral changes the causes or effects of endocrine changes?". Two different and testable models are summarized in Figure 21. The first model proposes that gonadal steroid changes drive behavioral changes. The reverse is proposed in the second model. As above, these models may be too simple. Feedback effects and complex interactions are probably important, but simple models must again be rejected before complicated processes are invoked.

The objective of the present chapter was to relate the phenomenon of functional sex change in teleosts to general patterns of teleost and vertebrate sexuality through application of organization-activation theory. If such an approach withstands experimental scrutiny, it would strengthen the case for using teleost sex change as a model for vertebrate sex determination and development (Reinboth, 1988; Shapiro, 1990). An understanding of sex change should enhance our understanding of not only the physiology, but perhaps also the evolution of sexual development generally.

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